TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

DONG LIN

NEW GENERATION AFFIBODY MOLECULE PHAGE LIBRARY FOR HIGHLY STRINGENT BINDER SELECTIONS

Master of Science Thesis

Examiner: Professor Matti Karp
Professor Per-Åke Nygren (KTH)
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ABSTRACT

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Affibody is a new class of engineered affinity protein, and has great properties compared with antibody. The aim of the project is to construct a new generation Affibody molecule phage library in which the bound phages are cleaved by trypsin in the selections against target molecules. First, a new phagemid vector pAffi-100-Tryp was created for new library preparation. Second, in comparative test selections with low-pH acid and trypsin cleavage elution, the functional display was ensured. Then, a new generation Affibody molecule library was constructed for highly stringent binder selections. The size of the library is approximately 4.7×10^9 cfu. In the selection against rhEphrin-B3, the bound phages were able to be cleaved by trypsin, and re-infect. After four rounds selection, candidate binders were selected for subcloning. According to the sequencing results, there were similar patterns among those candidate binders. Also, only Ile was found in Position 31.

PREFACE

This topic of the thesis project is related to the work I have done from April to August in the year of 2013 when I was a guest student in the research group "Affinity Protein Engineering" of Professor Per-Åke Nygren in the School of Biotechnology in Royal Institute of Technology (KTH), Sweden.

I would like to thank Prof. Per-Åke Nygren and MSc. Feifan Yu for supervising this work. My supervisor F.Yu designed most of the experiments and experimental protocols. She spent so much time to teach me during these five months, or I could not have completed the project without her. Also, I am grateful to all the colleagues whom I have had pleasure to work with in Division of Protein Technology in KTH. Furthermore, I would like to express my gratitude to Prof. Matti Karp and MSc. Suvi Santala who give quite valuable comments and suggestions on my thesis. Finally, I want to thank my family and friends for their supports.

Tampere, 9 March 2014

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LIST OF ABBREVIATIONS

ABD	Albumin Binding Domain
Amp	Ampicillin
BSA	Albumin from bovine serum
cfu	Colony-forming units
ELISA	Enzyme-linked immunesorbent assay
e-o-e	End-over-end
Ig	Immunoglobulin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Km	Kanamycin
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 1% (v/v) Tween
PCR	Polymerase chain reaction
PEG	Polyethylene glycol 6000
pfu	Plaque-forming units
RT	Room temperature
scFv	Single-Chain Variable-Fragment
ssDNA	Single stranded DNA
Tet	Tetracycline
TSB	Tryptic Soy Broth
TSB-Y	TSB supplemented with Yeast Extract

1. INTRODUCTION

Antibodies are still the most successful affinity proteins for life science applications, which is due to their capability to be virtually isolated and bind targets with high affinity and specificity. The most popular used antibody is IgG molecule. However, it has poor heat stability, also the manufacturing process is comparatively difficult and expensive (Löfblom *et al.*, 2010).

Affibody is recognized as a new class of engineered affinity protein, which is derived from the B domain of staphylococcal protein A (SPA). When compared with antibodies, Affibody molecules consist of alpha helices and lack disulfide bridges. In addition, Affibody owns higher physical and chemical stability. Affibody can be engineered and bind to a large number of target proteins or peptides with high affinity. There are 13 amino acid positions which can be randomly mutated in helix1 and helix2 of B domain to construct an Affibody library (Nygren, 2008). A specific Affibody is able to be obtained after affinity panning (Russel *et al.*, 2004). It is possible to co-express Affibody with reporter molecules such as fluorescent protein (Engfeldt *et al.*, 2005). Affibody has been used in detection, separation, and purification of target proteins (Löfblom *et al.*, 2010).

There are several commonly used Affibody selection systems: phage display, cell surface display, and ribosome display (Gunneriusson *et al.*, 1999; Friedman *et al.*, 2007; Grimm *et al.*, 2011). These different selection systems are associated with different aspects of protein expression and presentation. Phage display technology is the most widely used (Grimm, 2011). This technology is based on the filamentous bacteriophages with the ability to infect *Escherichia coli* cells. The foundation of this technology is the physical linkage between phenotype and genotype. It is possible to generate relatively large libraries and no need of costly reagents and equipment (Russel *et al.*, 2004).

The aim of the project is to construct a new generation Affibody molecule library for highly stringent binder selections. The project is performed in four stages: 1) phagemid vector engineering, 2) comparative test selections with low pH acid and Trypsin, 3) construction of large naïve Affibody library with phagemid pAffi-100-Tryp, and 4) selection to rhEphrin-B3. This project is supervised by Ph.D student Feifan Yu in Prof. Per-Åke Nygren research group: "Affinity Protein Engineering" in Royal Institute of Technology (KTH), Sweden from April to August 2013.

2. BACKGROUND

2.1 Protein engineering

2.1.1 Brief history

Proteins are polymeric compounds made up of amino acids. Amino acids are linked together via peptide bonds between the α-carboxyl groups and the α-amino groups. Generally, proteins refer to the structures containing 50 to 1000 amino acids per chain, while others possessing less than 50 amino acids are called peptides or polypeptides. Proteins play key roles in the process of life, and have diverse functions in all biological systems. For example, some proteins act as enzymes and catalyze specific biochemical reactions. Antibodies, serving as defensive proteins, can identify and bind to specific foreign molecules. Structural proteins support cell structure. Actin and myosin are contractile proteins which are fundamental for movement in muscles. Signal proteins are associated in regulation of cell metabolism. Transport protein hemoglobin transports oxygen (Ewait *et al.*, 2014).

Berzelius was the first person to coin the term "protein" in 1838. He considered this kind of material was the primitive substance of animal nutrition prepared by plants for the herbivores (Hartley, 1951). In 1926, Sumner revealed that urease is a protein by examining the crystallized form. He was the first one to demonstrate most enzymes are proteins, and got the Nobel Prize in chemistry in 1946 (Karplus *et al.*, 1997). The first sequenced protein is insulin which contains 51 amino acids. Sanger showed the amino acid sequence of insulin in 1951, and was rewarded the Nobel Prize in chemistry in 1958 (Sanger and Tuppy, 1951). Also in the year of 1958, the protein structure of myoglobin and oxy-myoglobin were demonstrated by X-ray crystallography (Kendrew *et al.*, 1958; Perutz *et al.*, 1960). The work was performed by Kendrew and Perutz, and they shared the Nobel Prize in chemistry in 1962.

Before 1980s, the only way to engineer protein was chemical modification with which nucleophilic amino acid side chains were modified with acylating or alkylating chemicals. In 1982, the tyrosyl-tRNA synthetase was the first enzyme studied in protein engineering. The tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* was engineered by systematic site-directed mutagenesis to substitute single amino acid residues. The result showed important experimental evidence about the enzyme catalysis theories (Greg *et al.*, 1982). This method enabled scientists to improve or alter existing protein traits according to application requirements.

However, rational approaches have limitations to predict the outcome of site-directed mutations. In order to overcome the restrictions, so-called library-based approaches were pointed out to engineer proteins (Griffiths *et al.*, 1994). Protein diversity can be generated via PCR. Mutations are randomly produced during amplification cycles with the diversity introduction in nucleic acids. Random mutants may be inserted into any piece of DNA by error prone PCR (Cirino *et al.*, 2003). The fidelity of the *Taq* DNA polymerase is modulated by altering the composition of the reaction buffer. Therefore, mistakes in base paring take place which results in the introduction of errors in the newly synthesized complementary DNA strand. Another method is to use cassette mutagenesis (Virnekas *et al.*, 1994). Mixtures of oligonucleotides are designed, synthesized, and then inserted into a plasmid. Both of the plasmid and the oligonucleotide are cleaved by the same restriction enzymes. The oligonucleotides containing the mutation are ligated complementarily into plasmid. This method enables almost 100% efficiency to generate mutants.

The possibility to engineer protein emerged with the rapid development of various studies on proteins. Protein engineering refers to the process of developing useful or valuable proteins. The generally used strategies can be classified into rational protein design and directed evolution (Grimm, 2007). In rational protein design, detailed knowledge of the structure and function of the protein is necessary to make desired changes. Site-directed mutagenesis is a typical rational design. However, it is extremely challenging to predict the effects of various mutations. Directed evolution mimics natural selection processes in a test-tube. Random mutagenesis is expressed in protein. The variants specific for a certain target are picked out in a selection system. Then, further rounds of mutation and re-selection are applied. Phage display is a very successful selection system. In 1985, Smith first displayed peptides on f1 filamentous phage. Phage display is based on the linkage of genotype and phenotype (Smith, 1985). It is a powerful system for protein selection. Afterwards, other selection systems such as bacterial display, ribosome display, and mRNA display were created (Gunneriusson *et al.*, 1999; Friedman *et al.*, 2007; Grimm *et al.*, 2011).

One important aim in protein engineering is to modify, create or inhibit protein-protein interaction. Affibodies have been designed to recognize pathogenic and foreign substances, and used as high-specificity reagents. Antibodies own the ability to bind specifically to their target molecule antigen. There are five different subclasses of antibody: IgG, IgM, IgA, IgE, and IgD. IgG is the most widely used antibody, and it consists of two identical heavy chains and two identical light chains with about 150 kDa molecular mass. Antibodies can be produced by immunizing animals with an antigen. Polyclonal antibodies with amino acid sequences are obtained, and they can recognize different epitopes on the same antigen. In 1975, Köhler and Milstein first introduced monoclonal antibody based on hybridoma technology (Köhler and Milstein, 1975). Monoclonal antibodies are produced by the fusion of antibody-producing B-cell and myeloma cancer cell. They only bind one epitope, and perform excellently as affinity proteins. However, antibodies are large complex multi-chain proteins with disulfide

cross-links. Various problems have been encountered which limit the development of some researches. New engineered antibody fragments, such as Fab fragment and scFv, are developed to overcome the restrictions. They have reduced size but still retain antigen binding specificity (Grönwall, 2008).

Alternative non-immunoglobulin affinity proteins have also been developed without the limitations of antibodies. New proteins that designed for novel binding functionality typically lack disulfide cross-links, have small size, good stability and solubility, and are easy to produce in large quantities in *E. coli*. The novel designed proteins generally have globular protein scaffolds or repeat protein scaffolds. Many globular protein scaffolds including monobodies, Affibody, PDZ, and Srchomology3 (SH3) as well as WW domains have been created to different binding specificities. Furthermore, some repeat proteins scaffolds are used in protein engineering, such as tetratricopeptide repeat (TPR), leucien-rich repeat (LRR), and ankyrin repeat (ANK) (Sawyer *et al.*, 2013).

2.1.2 Protein traits

2.1.2.1 Affinity and selectivity

Affinity and selectivity give the description of the protein binding characteristics. Binding to another molecule is one simple way for proteins to perform their function. The strength with which any two molecules bind to each other shows the affinity. The units of equilibrium constant (*K*) for a reaction where two molecules (A and B) bind to each other are liters/mole. *K* is calculated from the concentrations of A and B, as well as the concentration of AB at equilibrium:

$$K = \frac{[AB]}{[A][B]} \qquad (1)$$

where [A] and [B] are the individual concentrations of molecule A and B, and [AB] is the concentration of complex AB at equilibrium. *K* is also known as association or affinity constant K_a.

The equilibrium is maintained by a balance between the opposing reactions: dissociation and association, which is described as:

$$K = \frac{k_{on}}{k_{off}} \qquad (2)$$

where k_{off} is the dissociation constant, and k_{on} is the association constant. In order to get high affinities, both fast association constant k_{on} and slow dissociation constant k_{off} are needed. At equilibrium, the association rate is equal to the dissociation rate:

$$k_{on}[A][B] = k_{off}[AB] \qquad (3)$$

where $k_{on}[A][B]$ gives the association rate, and $k_{off}[AB]$ means the dissociation rate. The relationship between the free-energy change ΔG, and the equilibrium constant *K* is described as:

$$\Delta G = -0.00458\, T \log K \qquad (4)$$

where ΔG is in kilocalories and T is the absolute temperature in Kelvins. It is noted that for every 1.14 kcal/mole decrease in free energy the equilibrium constant increases by a factor of 10 at 37˚C (Alberts *et al.*, 2008) .

Selectivity is another import trait for protein generated for binding other molecules. It is the ability of a molecule to discriminate a particular analyte from a complex mixture without interference. Both of affinity and selectivity of a protein can be improved. For example, a variant of Herceptin was isolated with the ability to interact with human epidermal growth factor receptor 2 (HER2) and vascular endothelial growth factor (VEGF) at the antigen binding site (Bostrom *et al.*, 2009). Moreover, a conventional IgG molecule MEHD7945A was generated to bind two epitopes human epidermal growth factor receptor 3 (HER3) and human epidermal growth factor receptor (EGFR) with high affinity. It was demonstrated to inhibit both EGFR- and HER3 mediated signaling *in vitro* and *in vivo* (Schaefer et al., 2011).

Equilibrium dissociation constant K_d (1/ K_a) is frequently used to evaluate the affinity of a protein. In order to measure K_d, protein A with a fixed concentration is titrated with its ligand B in various concentrations. The bound complex AB gives a signal which correlates with its concentration. This approach has been applied in many techniques, such as isothermal titration calorimetry (ITC), enzyme-linked immunosorbent assay (ELISA) (Bobrovnik, 2003; Freyer and Lewis, 2008). However, there are some limitations of these techniques. The kinetic parameters k_{on} and k_{off} are not measurable, and it may take longer time to reach equilibrium in high affinity interactions.

Biospecific interaction analysis (BIA) is an optical technology which has been developed to study the binding properties of biomolecules. Biacore is a commercial optical biosensor system, and the detection principle is based on surface plasmon resonance (SPR). It enables to detect and measure the kinetic parameters of the interaction between two or more molecules. One advantage of this technology is that non-label of the molecules to detect is required. It also brings the possibility to measure the rate constants k_{on} and k_{off} to calculate K_d without the need to reach equilibrium. Surface plasmons are one kind electromagnetic wave, and they arise when the entering photons interact with oscillations of electron plasma in the metal. The energy of the incident photons must exactly match the energy of the plasmon. Total internal reflection of light on the metallic surface leads to the excitation of plasmons, where energy from incident photons is effectively transferred to plasmons. The most conventional SPR sensor is in Kretschmann configuration (Figure 1). The excitation of plasmon depends on the wavelength of the light λ, angle of incidence θ, and thickness of the metal film, as well as dielectric function ε of the metal, prism and medium adjacent to the metal film (Buerk, 1993; Roh *et al.*, 2011).

In practical, one component of ligand A or protein B is immobilized on the gold surface. When the other component flows over, proteins interaction occurs. The absorbed molecules cause a proportional increase in refractive index, which leads to a shift of incident angle in SPR. The kinetic formation of complexes (AB) can be expressed as:

$$\frac{d[AB]}{dt} = k_{on}[A][B] - k_{off}[AB] \qquad (5)$$

where A represents the ligand and B the analyte protein. k_{on} is the association constant, and k_{off} is the dissociation constant. t is the reaction time. After a certain time, the system gets a stable state when $k_{on}[A][B]$ equals $k_{off}[AB]$. The equilibrium constant K is described in *Eq.(2)*.Then, buffer without analyte flows the fluidic channel, and a desorption of the ligand occurs. In this phase, the kinetic equation is quite similar to the one in association. At last, a pulse of dissociating agent is injected to remove the remaining ligands and regenerate the free receptors (Figure 2) (Cooper, 2002; Berthier and Silberzan, 2010).

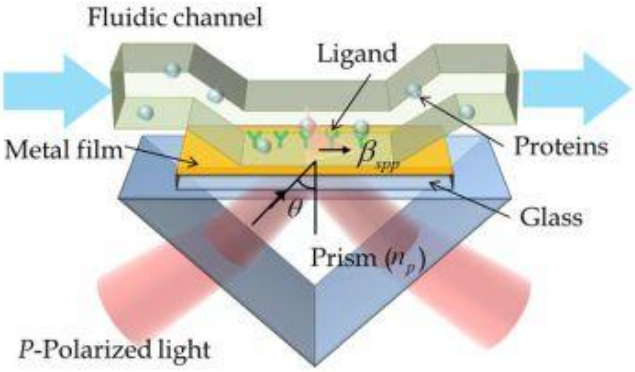

Figure 1. SPR sensor in Kretschmann configuration. In Kretschmann method, a prism coupler is attached with a thin metal film (Derived from Roh *et al.*, 2011). Targets protein flows over the immobilized ligands in fluidic channel. *P*-Polarized light enters with the incident angle θ. n_p is the refractive index of the dielectric prism. β_{spp} is the excited SPP (surface plasmon polariton) propagation constant.

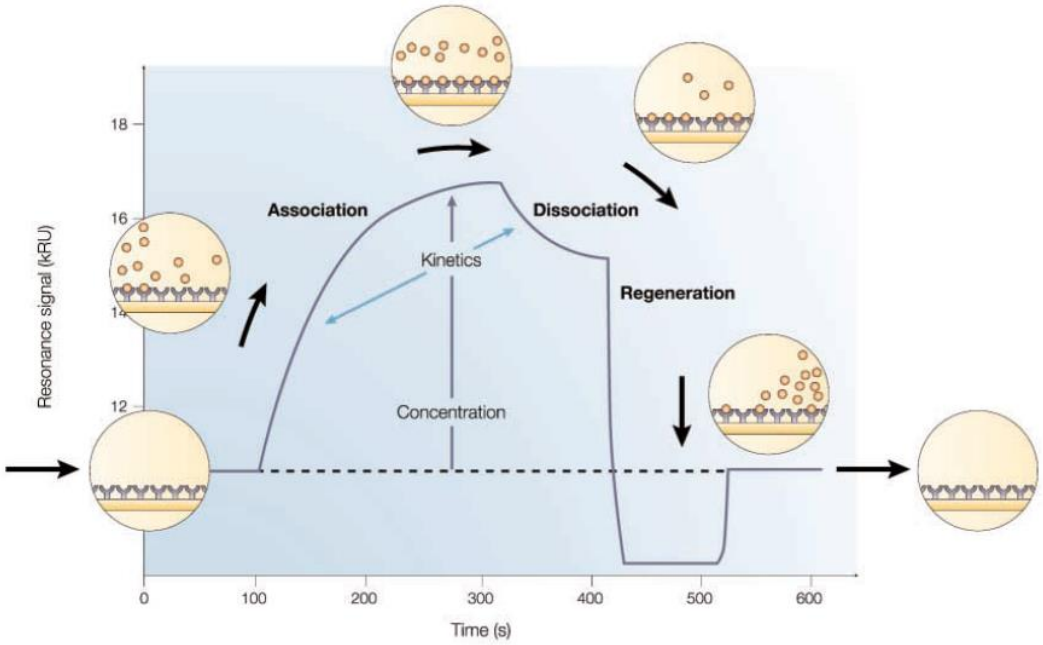

Figure 2. Binding kinetics in a typical binding cycle obtained by SPR (Derived from Cooper, 2002). Ligands (A) are immobilized on the sensor surface. With the injection of analytes (B), the resonance signal increases due to the formation of ligand-analyte complexes (AB). After the association phase, analytes solution is replaced by buffer. The dissociation of the complexes (AB) causes decrease in response. Then, a regeneration solution is used to disrupt binding and regenerate the surface.

2.1.2.2 Stability

Stability is the state of protein with net balance of forces. Protein stability normally refers to the physical thermodynamic stability. As for chemical stability, it involves loss of integrity caused by bond cleavage. Generally, the degradation processes take place in two steps. The first step is reversible which corresponds to the unfolding process, and the second step is irreversible which leads to the degradation of the unfolded protein molecule:

$$N \rightleftarrows U \rightarrow F \qquad (6)$$

where N represents the native proteins, U the unfolded state and F the final state. The stability of a protein refers to the difference in free energy between the native and unfolded state. *K* is defined as the equilibrium constant, it is the ratio of forward reaction $N \rightarrow U$ rate constant and reverse reaction $N \leftarrow U$ rate constant. It suggests the unfolding state of proteins is transient (Lumry and Eyring, 1954; Cueto *et al.*, 2003).

Extreme pH can lead to denaturation of some proteins. Electrostatic repulsion due to the titration of all the ionizing groups overcomes the attractive forces and results in unfolding of the protein. Denaturants, such as urea and guanidinium hydrochloride in high concentrations, can also affect the protein stability, and may cause complete unfolding of the protein (Grimm, 2007).

Temperature-mediated denaturation is related to the disruption of hydrogen bonding and the increase of hydrophobicity. Differential scanning calorimetry (DSC) is one frequently used method to analyze stability. DSC connects temperature with specific physical properties of substances, and it is the only method enables a direct determination of the enthalpy in the process of interest. The calorimetric changes in proteins are measured as a function of temperature. There is a difference between the heat capacity of the sample (C_p) and the one of reference buffer. Therefore, more energy is required for the sample to the same temperature as the reference. The additional energy is proportional to the excess heat capacity. Melting temperature T_M can be directly measured by DSC, it shows the temperature at which half of the protein molecules are in the native state and the other in the unfolded state. C_p of protein molecules is not constant in the process, and it gets increased for denaturation. The heat capacity C_p in unfolded state is greater than in folded state. The change of C_p is considered due to the disruption of the forces stabilizing native protein structure. Both equilibrium and kinetic of protein thermal stability can be quantified by DSC. DSC has the potential to become a universal method to study termal denaturation, because it does not rely on changes in spectroscopic signal (Grill *et al.*, 2010; Johnson, 2013).

Any technique which is able to distinguish proteins between unfolded state and folded state can be used to analyze stability. Another commonly used method is circular dichroism (CD). CD is based on the measurement of a very small different refractive indexes, when left and right circularly polarized light are simultaneously pass through the solution containing protein samples. CD spectra ranging from 160 to 250nm are usually used to analyze the secondary structures of proteins, while others with range

from 250 to 350nm are applied detect the side chain tertiary structures of proteins. In the thermal stability detection by CD, a single wavelength is chosen to monitor a certain specific structure. The signal at this wavelength is monitored with constant increasing temperature. An ellipticity value in degree is given corresponding to a certain temperature. In a typical graph of CD measurement, the midpoint of the transition shows the melting temperature T_M, and the width is related to the enthalpy of unfolding (Berova *et al.*, 2000; Kelly *et al*., 2005).

2.1.2.3 Enzyme activity

Probably, catalysis is the most important of all the functions of proteins. Enzymes are able to increase a reaction rate by a factor of up to 10^{20} over uncatalyzed reactions. In the Michaelis-Meten model (single substrate enzyme catalyzed reaction), the initial formation rate of product can be expressed as:

$$V = \frac{k_{cat}[E]_T[S]}{K_M + [S]} \qquad (7)$$

where V is the initial rate of reaction, [S] represents the substrate concentration, k_{cat} is the turnover number, K_M is called Michaelis constant, and $[E]_T$ gives the total concentration of enzyme. The turnover number k_{cat} is defined as the number of moles of substrate that react to form product per mole of enzyme per unit time, and it gives a dramatic illustration of the efficiency of enzymatic catalysis. If all the enzymes are saturated with substrate and the reaction occurs at the maximum rate, the formula can be written as:

$$V = \frac{V_{max}[S]}{K_M + [S]} \qquad (8)$$

where V_{max} is the maximum theoretical rate at infinite substrate concentration. When the rate of the reaction V is half its maximum value V_{max} , the substrate concentration [S] is equal to the Michaelis constant K_M (Campbell *et al.*, 2012).

An enzyme's activity refers to the general catalytic properties of the enzyme. The factors (e.g. substrate concentration, pH, and temperature) can affect the activity of an enzyme. In Michaelis-Menten formula, the measured rate varies more rapidly as the substrate concentration decreases. The reaction occurs with first-order kinetics, where the reaction rate depends on concentration of substrate. However, zero-order kinetics in the reaction is observed, if the enzyme is saturated with higher substrate concentration (well beyond $10 \times K_M$). It means the reaction rate dos not depend on concentration of substrate. Moreover, there are some enzymes known as allosteric, it shows a sigmoid shape of the rate variation with substrate concentration. Enzymes are active only within a limited range of pH. The maximum activity is obtained at an optimum pH value. The pH effect test is generally performed at high substrate concentration, because K_M might cause a change of initial reaction rate V. Generally, a pH value is chosen close to the optimum, also enables the operation of all other components in the assay. Temperature also influences enzyme's activity. 4-8% increase of reaction rate is caused by per degree

C growth. However, the enzyme protein denatures at high temperature, and lead to the decrease of product formation (Scopes, 2002).

Enzyme activity measurement is normally performed *in vitro* under defined conditions. In order to measure the activity of an enzyme, either how much product is formed over a given time or how much substrate has been utilized should be measured. There are two normally used approaches: stopped assay, and continuous assay. In stop assays, the reaction is stopped after a fixed time, and the amount of formed product is measured. The stop of the reaction is achieved by the denaturation of the enzyme. For example, strong acid, alkali or detergents are used, as well as irreversible inhibitors. Also, the addition of complexing agent such as EDTA removing the essential metal ions for activity can stop the reaction. Sometime, a separation step is required to separate remained substrate from product. The separation methods include chromatographic, solubility and partition procedures. If a selective method is available to distinguish between substrate and product, the separation step is not essential. The reaction is not disturbed in continuous assays and it is possible to check the results immediately. The amount of formed product is detected by measuring the change of some possible physical parameters when the reaction occurs, such as UV/vis absorption fluorescence emission or phosphorescence. For an instance, hydrogen peroxide (H_2O_2) and gluconate are the products by incubating glucose with glucose oxidase. With the help of peroxidase, H_2O_2 oxidizes a reduced dye and causes a color change (Scopes, 2002; Grimm, 2007).

2.2 Protein selection systems

2.2.1 Phage display

Bacteriophages are the viruses that can infect bacteria, and harmless to humans. There are countless types of bacteriophage (phage). Phage display is regarded as a means to display foreign proteins on filamentous bacteriophage. Filamentous bacteriophages contain a circular ssDNA genome which packaged into long filaments. These phages are non-lytic phages. Therefore, they are secreted into the environment without killing the host. Filamentous bacteriophages are specific for the Gram-negative bacteria *E. coli* having F-pili. The Ff family of filamentous bacteriophage includes f1, fd, and M13, which can infect F^+ *E. coli* through binding with their F pili (Bratkovič, 2009). In the life cycle of filamentous bacteriophages, they first infect the *E. coli* cells with F pili. After the infection, the ssDNA of phage is inserted into the cell and replicated into dsDNA. The genes encoding for the capsid proteins are transcribed and translated. New phages are assembled with new ssDNA and capsid proteins in the bacterial envelope, and hundreds of phages are released (Barbas *et al.*, 2004).

Phage display is used to express foreign (poly)peptides on the surface of phage particle. This is achieved by splicing a gene encoding into a gene encoding a capsid structural protein. It makes the linkage of genotype and phenotype of select recombinant proteins. Phage display technology was first described by George P. Smith in 1985. It

was reported that a foreign DNA fragment was inserted into the phage genome, and phage particles displaying foreign polypeptide as a fusion to the coat protein was yielded (Smith, 1985).

Foreign peptides and proteins can be displayed on the phage surface by fusing the gene to the phage ssDNA. M13 is the most commonly used filamentous bacteriophage. The ssDNA of M13 is encapsulated with about 2700 copies of major coat protein pVIII. There are five copies of pVII and pIX in one end, and five copies of pIII and pVI in the other end. The foreign proteins are displayed either on pIII or on pVIII. The basic principles of pIII and pVIII are quite similar. The perhaps reason for using either of the two capsid protein as the display platform is the display valency; peptides fused to pIII can be displayed in up to five copies per virion, while it is possible to present hundreds or thousands of peptides fused to pVIII in single phage (Bratkovič, 2009).

The most commonly used coat protein is pIII for display. There are three domains in g3p: N1, N2, and CT (C terminal) (Figure3). Each domain is separated by glycine-rich linker regions (Gly1 and Gly2). The N1 and N2 regions interact with each other intramolecularly. During the infection process, the N2 domain interacts with the F pilus, while the N2 domain forms a complex with the C-terminal domain of TolA at later stages. This interaction causes the release of the N1 domain from the intramolecular interaction with the N2 domain. Both of N1 and N2 domains are essential for the phage infectivity. Furthermore, the N-terminal part of p3 also prevents infected cell superinfection by another filamentous phage. The CT domain is buried in the phage particle and takes part in the final stages of virion assembly on inner bacterial membrane (Russel *et al.*, 2004; Bratkovič, 2009).

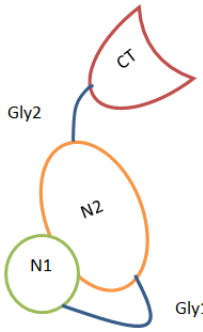

Figure 3 Schematic representation of minor coat protein pIII structure (Adapted from Russel *et al.*, 2004). From N- to C-terminus, the domains of pIII are N1, N2, and CT which are linked with glycine-rich linker regions.

If only the gene encoding for the foreign protein is inserted after the gene for pIII in the ssDNA of phage, it is called type 3 display. In the type 3 display, one copy of the gene of pIII is inserted, and five copies of foreign proteins are displayed. In order to reduce the copy number of displayed foreign protein, two modifications are often made. One is type 33 display, both gene encoding for the wild-type pIII (without fusion) and gene encoding for the pIII fused with foreign protein are inserted in the ssDNA. Therefore, there are both wild-type pIII and pIII with fusion protein exist on the new produced phage. The other one is type 3+3 display, a special display vector called a

phagemid was introduced (Figure 4). Phagemid have the both features of plasmid and phage vector. It carries antibiotic resistance and enables replication of dsDNA, also it allows for production and packing of ssDNA into virions. Phagemids are engineered to express recombinant pIII fusions under controlled conditions. Helper phages, which belong to Ff phage, are essentially required in the type 3+3 display. They contain the gene encoding for all the remaining phage proteins which are essential in phage particle produce, and a compromised origin that leads to inefficient packaging. With infected by helper phage, the bacterial host having phagemid can express the wild-type phage proteins from the helper phage genomes and a small amount of the fusion protein encoded by the phagemid (Bratkovič, 2009).

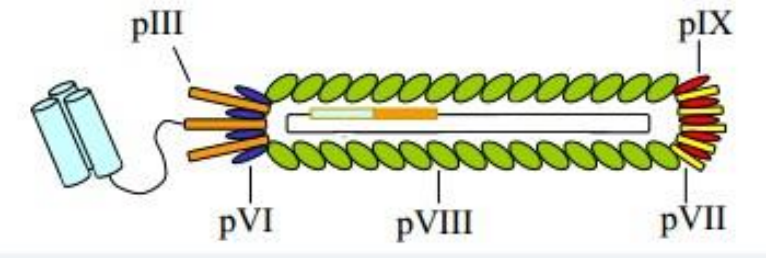

Figure 4 Schematic view of a M13 filamentous bacteriophage from phagemid pAffi-100-Zlib-Tryp (Modified from Hedberg, 2003). The phage particle contains phagemid vector pAffi-100-Zlib-Tryp which is surrounded by several different coat proteins. Foreign protein - affibody- is displayed and fused on full-length pIII.

Potential binders may be selected for their binding properties from a large library. A typical phage display selection cycle is shown in Figure 5. The selection process is achieved by incubating the phages particles to immobilized targets and then washing away unbound phages. The selection pressure is influenced by the antigen concentration, washing times and concentration of a competitor antigen added. Bound phages are eluted via low pH, high pH, or enzymatic cleavage. The eluted phages have the ability to infect *E. coli* cells and get enriched with helper phage. This process is also called phage panning. After several rounds of enrichments, the potential binders could be selected (Bradbury and Marks, 2004; Grimm, 2007).

Because of the presence of non-selectively binding phages, more than one selection cycles are required. The selection pressure increases in the consecutive selections. Furthermore, a pre-selection is performed in order to avoid undesired binding phages. The phage library is loaded to the solid support without the target, before exposed to the target molecule. The concentration of phage is evaluated by the titers, so it shows the effect of selection and enrichment of binding phages after phage production. In the post-selection analysis, the selected clones are screened for the binding properties. Afterwards, the most promising candidates are prepared for further traits test (Bradbury and Marks, 2004; Grimm, 2007).

Since phage is with high robustness and few costly laboratory reagents or equipment is required, phage display is the most widely used selection system.

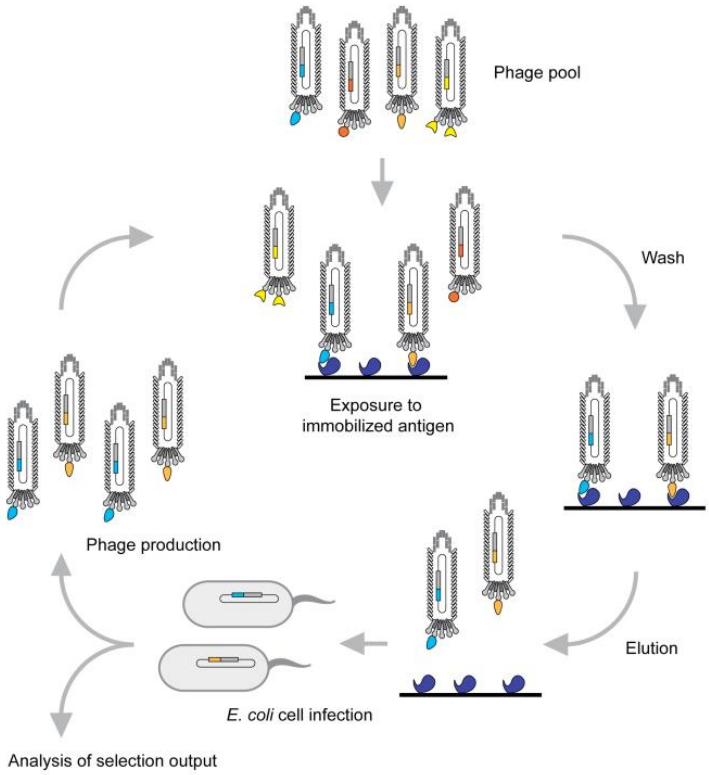

Figure 5. Illustration of a typical phage display selection cycle (Derived from Grimm, 2007). Initially, library containing phage pool is exposed to immobilized antigen (target). Then, non-bound and weakly-bound phages are removed in wash steps. Bound phages are eluted off and used to infect E-coli cell. The infected cells are stored for post-selection analysis or for phage produce before a new selection cycle

2.2.2 Cell surface display

In cell surface display, foreign peptides and proteins of interest are expressed on the surface of microbial cells. It is allowed to monitor the phenotype of the cell during selection using quantitative flow cytometric analysis (Grimm, 2007). The protein to be displayed can be fused to a carrier protein by N-terminal fusion, C-terminal fusion or sandwich fusion (Lee *et al.*, 2003; Grimm, 2007).

Gram-negative bacteria surface display

Gram-positive bacteria have a complex cell envelope structure consisting of the cytoplasmic membrane, periplasm and outer membrane. Therefore, the proteins need to cross two membranes and reach the extracellular milieu (Lee *et al.*, 2003).

The N-terminal fusion approach is used when there is a directing and anchoring domain in the C-terminus of the carrier protein. Peptidoglycan-associated lipoprotein (PAL) is regarded as a good example of the N-terminal fusion approach. The C-terminal portion of PAL binds to the peptidoglycan layer, while the N-terminal cysteine with lipid moiety modification binds to the outer membrane (Dhillon *et al.*, 1999). The C-terminal fusion method is applied when the carrier proteins containing targeting se-

quences on their N-termini. One typical carrier protein of this type is the Lpp-OmpA hybrid. The Lpp part is responsible for the proper localization to the outer membrane, while the OmpA part is related to the transportation of foreign proteins (Richins *et al.*, 1997; Lee *et al.*, 2003). The most commonly used approach is the sandwich fusion for Gram-negative bacterial surface display. The carrier proteins used in this method can be divided into three classes: outer membrane proteins (OMPs), S-layer proteins, and subunit proteins of extracellular appendages (Lee *et al.*, 2003). OMPs form transmembrane β–barrels on the outer membrane. The short loops connecting antiparallel β-strains on the external side can potentially be used as fusion sites for foreign peptides and proteins. In one research, the peptide containing 162 amino acids was fused to *E. coli* OmpC (Xu and Lee, 1999). S-layer protein from *Caulobacter crescentus* possesses N-terminal domain binding to the outer membrane, and C-terminus having a secretion signal (Bingle *et al.*, 1997). In *E. coli*, the subunit proteins of flagella and fimbriae can be applied to display foreign proteins. The subunit proteins attached by heterologous sequences are still possible to assemble into polymetric appendages (Westerlund-Wikström *et al.*, 1997; Stentejerg-Olesen et al., 1997)

E. coli is one of the most commonly used Gram-positive bacteria host, it is an ideal host for screening a large peptide or protein library. It is possible to get 5×10^4-10^5 copies of library members displayed on a single *E. coli* cell. If the cell incubated with an antigen with fluorescent label, the fluorescence signals detected by flow cytometry are correlated with the affinity and the copy number of the displayed library members on a cell (Francisco *et al.*, 1993). However, the fragility of outer membrane is still a problem due to the display of proteins (Lee *et al.*, 2003).

Gram-positive bacteria surface display

Gram-positive bacteria have a single cell membrane and a thick layer of proteoglycan. Proteins displayed must be attached to the cell envelope when Gram-positive bacteria are used as host for the surface display. They can be linked either to the cytoplasmic membrane or cell wall components. Four major types of displayed proteins were discussed in the review by Desvaux and his colleague, they were a) proteins anchored to the cytoplasmic transmembrane, b) lipoproteins covalently attached to the membrane lipids after cleavage by signal peptidase II, c) proteins possessing C-terminal LPXTG-like (in single-letter amino acid code, where X denotes any amino acid) motif and covalently attached to peptidoglycan by sortase, and d) proteins recognizing some cell wall binding domains (Desvaux *et al.*, 2006).

Staphylococcal protein A (SPA) has been used as a model to analyze the anchoring mechanisms of surface proteins, and it contains LPXTG-like motif followed by a hydrophobic domain and a charged tail. The LPXTG can be cleaved by sortase (SrtA) in the polypeptides between the threonine and the glycine. The hydrophobic domain serves as a membrane-spanning region, and the charged tail is regarded as a retention signal to prevent the peptide chain secreting out to the surrounding medium (Scheewind *et al.*,

1993). S-layer homology domain (SLHD) proteins possess cell wall binding domains. SLHDs are present as 1-3 SLD modules/motifs at the N-terminus of Gram-positive S-layer proteins. S-layer proteins are able to bind very strongly to the cell wall via non-covalent interactions (Mesnage *et al.*, 1999).

Staphylococcus carnosus is a food-grade bacterial strain, and it has been used for Gram-positive bacterial surface display. In 1995, Samuelson and co-workers conducted a display of heterologous proteins on *S. carnosus* cells. It was also the first time to utilize fluorescence-activated cell sorting to analyze the presence of surface-displayed hybrid receptors on Gram-positive bacteria (Samuelson *et al.*, 1995). Because of the significant increase in transformation efficiency, it was allowed for the display and screening of the first libraries (Löfblom *et al.*, 2007). The first high-complexity protein library displayed on the surface of Gram-positive bacteria was constructed in the year of 2007 (Nina *et al.*, 2008).

Yeast surface display

Yeast surface display was introduced by Boder and Wittrup research group in 1997. In the report, the selection of scFv antibody fragments against three-fold decreased antigen dissociation rate from a library displayed on Saccharomyces cerevisiae was performed. The library was fused to the C-terminus of the mating adhesion receptor (Boder and Wittrup, 1997).

There are two types of mannoproteins present in the cell wall of S. cerevisiae: sodium dodecyl sulfate (SDS)-extractable and glucanase-extractable mannoproteins. The SDS-extractable mannoproteins are non-covalently bound to the cell wall, and they are able to be extracted by using SDS and reducing agents. The glucanase-extractable mannoproteins are linked to β-glucan of the cell wall via covalent bond, and it is possible to get them released by digestion with β-glucanase. Generally, the glucanase-extractable mannoproteins have a putative glycosyl phosphatidylinositol (GPI) attachment signal at the C-terminus (Van der Vaart *et al.*, 1997; Lee *et al.*, 2003). GPI anchor is required for the covalent association of these proteins with the cell wall due to the secretory pathway. The display of foreign peptide and protein is achieved by fusion to a mannoprotein. *S. cerevisiae* α-agglutinin, a-agglutinin, and flocculin has been used as an anchor protein (Kondo and Ueda, 2004). Almost all the yeast surface display applications are GPI anchor-dependent (Lee *et al.*, 2003).

There are unique advantages for yeast surface display. Yeast is a eukaryote, and it is recognized as safe in food and pharmaceutical applications. Also, it is able to display proteins with post-translational modification and having large molecular mass. It was found that yeast display could yield more high affinity clones compared to phage display, when using the same library towards the same antigen (Bowley et al., 2007). Furthermore, yeast display has become more and more popular since 1997. It was possible to improve the affinity of a scFv for fluorescein biotin to as low as 48 fM (Boder *et al.*, 2000).

2.2.3 Ribosome display

Ribosome display is a display system which does not rely on living cells to link genotype and phenotype. There are two main advantages for ribosome display: a) the diversity of the library is only limited by the number of ribosomes and different mRNA molecules, b) it is easy to introduce random mutations after each selection round (Zahnd *et al.*, 2007). The first description about ribosome display for peptides was reported by Mattheakis and co-workers in 1994 (Mattheakis *et al.*, 1994).

The concept of ribosome display is very simple to understand, and the core point is to translate an mRNA molecules library with stoichiometric quantity of ribosomes (Lipovsek and Plückthun, 2004). In principle, a library of DNA without stop codon is *in vitro* transcribed into the library of mRNA. Then, the mRNA library is *in vitro* translated. The translation of mRNA performed in cell extracts of *E. coli*, wheat germ or rabbit reticulocytes. *E. coli* S-30 extracts are the most frequently used for translation in ribosome display, because of containing ribosome-associated factors which are important for protein folding. Because of the lack of stop codon in mRNA, the ribosome will frequently get the very end of mRNA molecules. Also, the end of the newly synthesized polypeptide is still within the ribosomal tunnel, and its last amino acid is still connected to the peptidyl-tRNA. Therefore, a ternary complex is formed with mRNA, ribosome and the corresponding polypeptide, and the mRNA connects between the phenotype (the protein) and genotype (the mRNA). The complexes serve as library members and enter a selection cycle, where the principle is similar to the one in the phage display. After each cycle of selection, the complexes with binding targets isolated, and reverse transcribed into DNA which is followed by the PCR amplification. The new template is generated and prepared for the next selection cycle (Figure 6) (Lipovesk and Plückthun, 2004; Grimm, 2007; Hoffmann *et al*., 2010).

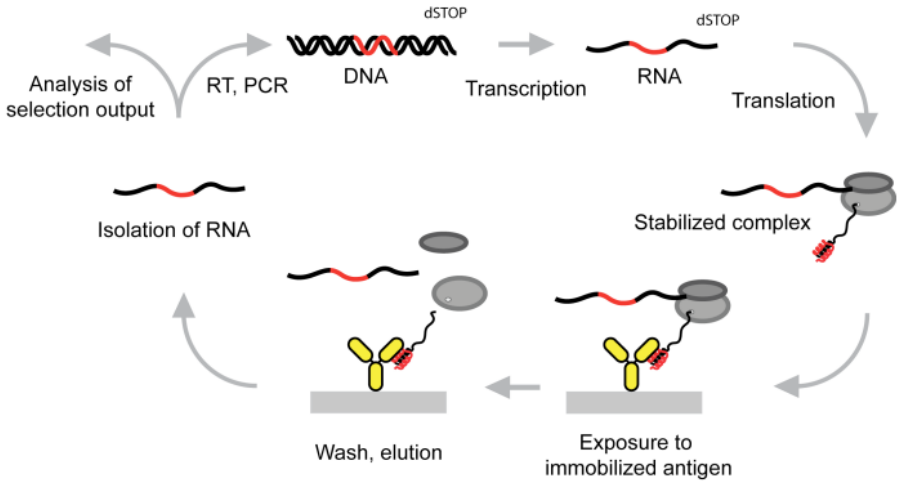

Figure 6. Illustration of a typical ribosome display selection cycle (Derived from Grimm, 2007). DNA encoding the library without stop codon is *in vitro* transcribed into mRNA. The mRNA library lack of stop codon is *in vitro* translated, and the complexes of mRNA-ribosome-protein are formed. Then, the stabilized complexes are exposed to the immobilized antigen. Non-binding complexes are washed away, and the binding ones are eluted and purified. Isolated mRNA is either reverse transcribed and amplified, or prepared for the analysis.

A typical structure of linear DNA template in prokaryotic ribosome display system is shown in Figure 7. T7 promoter is essential in the DNA template for the transcription, and it allows for mRNA synthesis. The followed ribosome binding site (RBS) is able to base-pair with ribosomal RNA, which is Shine-Dalgarno (SD) sequence. SD sequence is related to the initiation of translation. The gene for polypeptide to be displayed is inserted after RBS. A spacer protein is also needed for the fold flexibility of proteins. Generally, at least 23-30 amino acids are required as spacer protein (He *et al.*, 2005; Grimm, 2007).

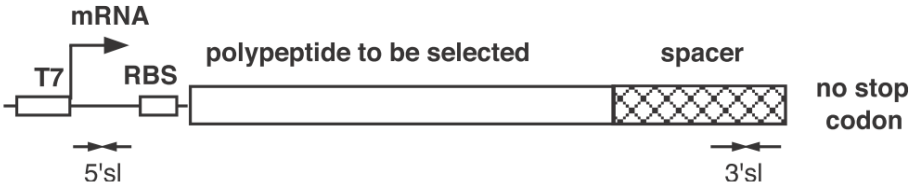

Figure 7. A typical linear DNA template in prokaryotic ribosome display (Derived from Lipovsek and Plückthun, 2004). T7 is the T7 promoter for the start of mRNA synthesis. SD serves as RBD. Spacer is attached after the polypeptide to display. 5'sl and 3'sl denotes the stem loops on the 5'- and 3'-ends of the mRNA respectively.

The first complex library tested for selection and affinity maturation by ribosome display was the one of antibody scFv fragments. The library was generated from immunized mice. It was able to functionally express scFv with good yields in the *E. coli* extracts, and the lowest dissociation constant of scFv could reach $(4\pm1)\times10^{-11}$ M (Hanes *et al.*, 1998). Afterwards, synthetic library was introduced into the ribosome display system. In the study, scFv from a large synthetic library was selected and evolved again bovine insulin, and their equilibrium dissociation constants are as low as 8×10^{-11} M. Also, error-prone PCR was used for point mutations in this study, and it allowed a 40-fold increase in affinity when compared to the progenitor clones (Hanes *et al.*, 2000).

2.3 Affibody molecule

2.3.1 Affibody technology

Affibody molecules consist of 58 amino acids and whose relative molecular mass is about 6.5 kDa. There are three α-helices in the structure (Figure 8). Affibody molecules were originally derived from the B-domain of SPA. In the G29 of B-domain, Gly was substituted by Ala. This engineered variant was called Z domain. The G29A mutation enabled Z domain to enhance chemical stability, and increase the folding rate constant by threefold as well. The engineered Z-domain still had the affinity for the Fc part of the antibody but almost lost the weaker affinity for the Fab region (Arora *et al.*, 2004; Löfblom *et al.*, 2010). Affibody molecules were initially described in 1996. These small binding proteins were achieved by randomization of 13 solvent-accessible surface residues of stable α-helical bacterial receptor domain Z (Nord *et al.*, 1996). As reported,

one of the highest affinity of an Affibody molecule is 22 pM for the breast cancer marker Her2 (Nord *et al.*, 2006).

Affibody molecules are a new class of affinity ligands, which have the similar functions to those of antibodies. They are regarded as a viable and sometimes superior alternative to antibodies. When compared with antibody, Affibody molecule is non-immunoglobulin-based, and the isolation is performed using synthetic combinatorial libraries and *in vitro* selection systems. Also, the frameworks for building Affibody molecules are smaller and structurally less complex than immunoglobulins, which brings the advantages to both production and application issues. Affibody molecules have the proven potential for therapeutic, diagnostic and biotechnological application (Löfblom *et al.*, 2010).

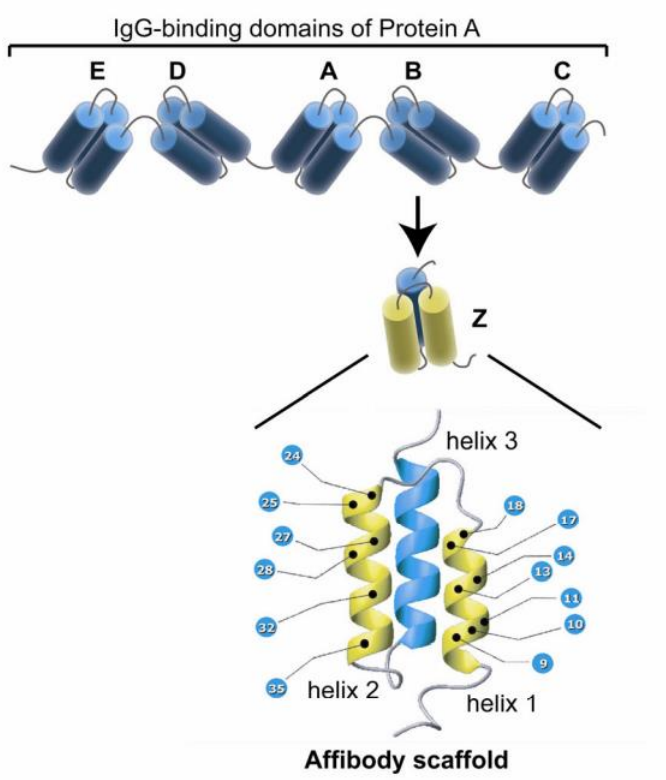

Figure 8. Schematic view of the Z domain of Affibody scaffold (Derived from Grönwall, 2008). Z domain is derived from the B domain of SPA which contains five IgG-binding domains. 13 surface-located amino acid residues in helices 1 and 2 have been used to create combinatorial protein libraries.

First-generation Affibody libraries have been constructed through the genetic randomization of 13 amino acids which are located on the surface of the Z-domain scaffold. The randomization positions are determined by the analysis of an available X-ray crystallography structure of the co-complex between the homologous B-domain of SPA and human IgG (Deisenhofer, 1981). These 13 positions are all in helices 1 (7 positions) and helices 2 (6 positions) of the three-helix bundle. Most of the 13 positions are related to the interaction with Fc region of human IgG1. With 32 codons and 20 amino acids, the theoretical diversity of genetic variants in a library is 32^{13} (3.7×10^{19}), and 20^{13} (8.2×10^{16}) protein variants can be encoded (Nygren, 2008). There are two general strat-

egies applied to increase the affinity of members selected from the first-generation library. One is to keep the highly conserved binding residues constant and randomize the remaining binding positions after post-selection analysis, and make a re-selection against the same target. The other one is construct multivalent Affibodies from a selected monomeric Affibody (Steffen *et al.*, 2005; Orlova *et al.*, 2006; Sawyer *et al.*, 2013). Second-generation Affibodies are designed by scaffold optimization. Site-specific mutagenesis of residues outside the binding surface was used to improve properties of Affibody molecule (Feldwisch *et al*., 2010).

Affibody can be obtained and enriched by *in vitro* selection. For example, phage display has been used to identify library members. A large scale of a specific Affibody is able to be produced chemical synthesis or prokaryotic expression (Rönnmark *et al*., 2002; Engfeldt *et al*., 2005).

2.3.2 Applications

2.3.2.1 Biotechnology

Affibody molecules have been used for targeting various proteins, and regarded as the capturing agents on affinity chromatography columns for protein purification. In one research, Affibodies were selectively bound to bacterial *Taq* DNA polymerase and human apolipoprotein A-1_M, respectively. It was possible to efficiently recover both target proteins from crude cell lysates when using these two Affibodies in affinity chromatography. Both column capacity and selectivity kept the same in repeated cycles of sample loading, washing and low pH elution (Nord *et al.*, 2000). Furthermore, Affibody has successfully applied for selective binding to human IgA. First, phage display technology was used to selective Affibodies binding to IgA monoclonal antibodies. It was shown that five out of eight selective Affibodies were capable of IgA after biosensor technology applied. The lowest dissociation constants (K_d) reached 0.5μM. Z_{IgA1} with strongest binding affinity also showed the ability to selectivity recognize human IgA subclasses (IgA_1 and IgA_2) and secretory IgA. In further analysis, Z_{IgA1} was constructed as divalent head-tail dimer in affinity column, and able to recover IgA protein from a spiked *E. coli* cell lysate in affinity chromatography (Rönnmark *et al.*, 2002). Another application showed Alzheimer amyloid β (Aβ) peptides binding Affibody. It was reported that selected Affibody molecules could efficiently capture Aβ peptides from human plasma and serum (Grönwall *et al.*, 2006).

Due to the small size and favorable biophysical properties of Affibody molecules, they have widely applied in other biotechnology areas traditionally dominated by antibodies. Various proteins have been linked to Affibody molecules which enabled facile detection of targets in assays. Affibody specific to surface protein G of respiratory syncytial virus (RSV) was genetically fused to the Fc fragment of human IgG, and led to Affibody-Fc chimeras. The chimeras were demonstrated to efficiently detect targets proteins in a complex background containing *E. coli* lysate in Western blotting experi-

ment (Rönnmark *et al.*, 2002). Affibody was also reported to link various enzymes. The enzyme β-galactosidase (β-gal) was conjugated with Affibody Z_{IgA1}, which resulted in two kinds of chimeras: Z_{IgA1}-β-gal and $(Z_{IgA1})_2$-β-gal. They were applied to detect IgA in enzyme-linked immunosorbent assay (ELISA) and dot-blot analyses (Rönnmark *et al.*, 2003). Fusing Affibody, targeting IgA and IgE, to the bacterial surface protein made it possible to use whole-cell analysis in agglutination assays or whole-cell immunoprecipitations (Gunneriusson *et al.*, 1999).

Affibody also serves as affinity probes in protein micro-array formats. In one study, dimeric Affiobdy molecules targeting IgA, IgE, IgG, TNFα, Insulin and *Taq* polymerase, respectively, were immobilized on thiol dextran microarray slides. After the slides incubated with fluorescently labeled, specific binding of respective target proteins was observed without cross-reactivity. The detection limit of the best performing Affibody was about 70fM (Renberg *et al.*, 2007). In the same research, Affibody molecules were also used as capture agents in a sandwich array format where unlabeled protein and monoclonal antibodies were specifically selected in serum or plasma sample (Renberg *et al.*, 2007).

Fluorescent molecules are able to couple to Affibody molecules. Oregon Green 488 dye was conjugated to a C-terminal cysteine of a HER2 targeting. Then, flow cytometry and immunefluorescent confocal microscopy were used to analyze HER2 expression levels on cancer cell lines and cryosections of SKOV-3 xenograft tumors (Lundberg *et al.*, 2007). Another similar research was focused on the HER2 and EGFR-specific Afffibody which fused to fluorescent proteins. The expression levels of HER2 and EGFR on cell lines and in tissue were measured via flow cytometry and confocal imaging (Lyakhov *et al.*, 2010).

Furthermore, Affibody molecules have been investigated as biosensor to detect analytes by fluorescence resonance energy transfer (FRET). Affibody molecules with the specific affinity for IgA and IgG, respectively, were labeled with EDANS and NBDX which were site-introduced at opposite ends of Affibody molecules. The donor/acceptor pair enabled FRET. As well, a biotin moiety was labeled on the Affibody for surface immobilization. Fluorescent-labelled protein was titrated with increasing concentration of protein, and it showed shifting relative emission of the fluorescent probes (Engfeldt *et al.*, 2005). Another research based on FRET for specific detection of target proteins used antiidiotypic Affibody pairs. Idiotypic target-specific Affibody was labeled with fluorescein, while antiidiotypic Affibody was labeled with tetramethylrhodamine. These two Affibodies were used in a homogeneous competitive binding assay, where intramolecular FRET between the two fluorescent probes was gained in the antiidiotypic Affibody complex. However, the FRET signal decreased with the addition of target protein, because antiidiotypic Affibody was displaced (Renberg *et al.*, 2004).

2.3.2.2 Imaging

Imaging is an important area where Affibody is applied to detect the lesions in the body. The radiolabeled Affibody has shown great advantages in single-photon emission spectroscopy (SPECT) and positron emission tomography (PET). The half live of Affibody is shorter than positron emitting nuclides and gamma emitting nuclides (Orlova *et al.*, 2006).

The cancer-specific cell-surface receptor HER2 was traced with HER2-binding Affibody for imaging. HER2 is a transmembrane protein, and belongs to the human epidermal growth factor tyrosine kinase receptor family. It was reported to overexpress in several cancer types, such as 23% to 30% in breast and ovarian cancers (Wang *et al.*, 2001). The detection of HER2 expression is suggested in the tumor lesions diagnosis. HER2-binding Affibody was selected out from the naïve phage library, and showed the great ability to bind to HER2 protein in SKOV- 3 which is a human ovarian carcinoma cell line. Moreover, studies in rats were performed. Affibody was observed to get enriched within one hour after the tail vein injection. Due to the rapid blood clearance, it was allowed to image tumors in mouse xenografts models in 30-60 minutes after injection. Furthermore, it was possible to label Affibody with ^{18}F ($t_{2/1}$ 110min), ^{68}Ga ($t_{2/1}$ 68min), ^{99m}Tc ($t_{2/1}$ 6h), and ^{111}In ($t_{2/1}$ 2.8 days) to extend the half live of Affibody. However, antibodies and scFv or Fab fragments are not well compatible with short-lived positron emitters, such as ^{18}F and ^{68}Ga. Also, Affibody has smaller size and faster biodistribution compared with antibodies and scFv or Fab fragments (Löfblom *et al.*, 2010).

Site-specific labeling of Affibody allows the clinical utilization. Labeling can be achieved by site-specific coupling of a chelator during peptide synthesis, or by incorporation of a unique cysteine and using thiol-directed chemistry (Löfblom *et al.*, 2010). The first site-specific radiolabeling Affibody molecule was $Z_{HER2:342}$, and it was produced via peptide synthesis. DOTA was attached to the N-terminal in the last synthesis step to yield DOTA- $Z_{HER2:342\text{-}pep2}$ (ABY002). Then, ^{111}In was labeled to ABY002. The formed ^{111}In-ABY002 was injected to the mice with SKOV-3 tumor. ^{111}In-ABY002 was efficiently distributed in the body and bound to the HER2 overexpressing tumor. It always showed higher uptake in tumor than in other organs expect kidney. High tumor to blood ratio enabled the imaging of tumors in 1h after injection (Orlova *et al.*, 2007). Another research using ^{68}Ga as radiolabel on ABY002 showed analogic results (Tolmachev *et al.*, 2010).

Affibody can be dual-labeled to make a site-by-site comparison of two different labels. For example, in the experiment of co-injection of ^{68}Ga- and ^{111}In-ABY002, it gave the result that the tumor uptakes for both molecules were quite similar. However, there was significant higher radioactivity of ^{111}In-ABY002 in blood, lung, spleen and the gastrointestinal tract. The first clinical experiment of ^{68}Ga- and ^{111}In-ABY002 was applied to three patients with metastatic breast cancer. It took only 2-3h to obtain high contract SPECT or PET images (Baum *et al.*, 2010).

11 amino acids in the scaffold of $Z_{HER2:342}$ were engineered to obtain new HER2-binding Affibody molecule $Z_{HER2:2891}$ which have higher hydrophilicity and higher thermal stability. Maleimido-DOTA was attached to the C-terminal cysteine, yielding [MMA-DOTA-Cys61]-$Z_{HER2:2891}$ - Cys (ABY025). It was reported that ^{111}In-ABY-025 bound specifically to HER2 both in vitro and in vivo (Ahlgren *et al.*, 2010). Another new molecule $Z_{HER2:342}$-ABD_{035}-DOTA (ABY027) was constructed by adding ABD_{035}, which had a high affinity for human serum albumin, to the N-terminal of $Z_{HER2:342}$ followed by the conjugation of DOTA to the N-terminal of ABD_{035}. ^{177}Lu was used as radiolabel for ABY027. At 48h after injection, tumor uptake of ^{177}Lu-ABY027 was as 3-fold as that for previously reported ABD-$(Z_{HER2:342})_2$. ABY027 was suggested more suitable for therapy (Tolmachev *et al.*, 2007; Jonsson *et al.*, 2008; Orlova *et al.*, 2013).

Moreover, Affibody is applied in magnetic resonance imaging (MRI) which is a powerful tool for in vivo anatomical and molecular imaging. Superparamagnetic iron oxide (SPIO) particles targeted with HER2 Affibody enabled the image of HER2 in murine tumor xenograft (Kinoshita *et al.*, 2010).

2.3.2.3 Therapeutic applications

One outstanding application of Affibody molecule is in therapeutic area. One hybrid protein, containing Affibody molecule as affinity recognition moiety and a toxic domain with therapeutic efficacy, is an outstanding payload. A novel recombinant Affitoxin was developed with the structure consisting of HER2-specific cytotoxic molecules and HER2-specific Affibody. The cytotoxic molecules were truncated from *Pseudomonas exotoxin* A (PE), which was a cell killing agent. In the report, Affitoxin could bind specifically to HER2 with nanomolar affinity, and it was demonstrated to treat HER2-positive tumors (Zielinski *et al.*, 2009).

The success of Affibody utilization in imaging supports essential knowledge for therapeutic applications. Distribution of radionuclides labeled Affibody molecules obtained from imaging enables the estimation of delivered dose and toxic effects. The calculated dose of $Z_{HER2:342}$ was tested by biodistribution studies with ^{111}In label. The result from the study suggested that ^{111}In-benzyl-DTPA-$Z_{HER2:342}$ was a promising candidate for detection of HER2 expression in malignant tumors (Tolmachev *et al.*, 2006). Another ^{186}Re-labeled HER2 specific Affibody conjugate maGSG- $Z_{HER2:342}$ was demonstrated promising for therapeutic application (Orlova *et al.*, 2010). The internalization of Affibody molecule into malignant cells has also been concerned. ^{111}In labeled DOTA-$Z_{HER2:342\text{-}pep2}$ was studied on three HER2-expressing cell lines: SKOV-3, SKBR-3, and BT474. It was found that both continuous and interrupted incubation of tumor cells with ^{111}In-DOTA- $Z_{HER2:342\text{-}pep2}$ made a slow internalization, while the tracer strongly binding to the cell-surface receptors enabled a high level of cellular retention (Wallberg and Orlova, 2008).

An albumin binding domain (ABD) was fused to a radiolabeled anti-HER2 Affibody. The introduction of ABD could interact with serum albumin, and enabled a 5-

fold increase in tumor uptake when compared with non ABD-fused Affibody. It was shown that ^{177}Lu labeled Affibody-ABD chimera displayed specific binding to HER2-expressing cells *in vitro,* and completely prevented formation of tumors when injected into mice with HER-2 expressing SKOV-3 ovary cancer microxenografts (Tolmachev *et al.*, 2007).

Liposomes can protect the elimination and degradation of encapsulated drugs, and they have been utilized with Affibody molecules. The cysteine-modified Affibody molecule targeting EGFR was chemically coupled to maleimide-PEG_{2000}-DSPE. Subsequently the conjugate was inserted into PEGylated liposomes, which led to stabilized Affibody liposomes (SALs). These SALs could strongly and selectively bind to EGFR-expressing tumor cell lines, also enable efficient internalization of the liposomes. The cytotoxicity to EGFR-expressing cells was imporved by using mitoxantrone-loaded liposomes (Beuttler *et al.*, 2009). In another study, Afffibody $Z_{HER2:342}$-Cys was conjugated with thermosensitive liposomes, and formed HER^+ Affisomes which retained the thermosensitivity (Puri *et al.*, 2008). In the following research, a spacer at the C-terminus of the Affibody molecule was inserted, in order to enhance the binding ability to cell surface-expressed HER2 (Smith *et al.*, 2011).

Affibody has also been investigated with human adenoviruses (Ad) which used as vector in cancer gene therapy. Human adenoviruses are engineered to remove the binding ability to native receptors. Adenovirus type 5 (Ad5) was re-targeted to HER2/neu by the insertion of a dimeric Affibody molecule ZH in the HI-loop (Magnusson *et al.*, 2007). Another Affibody molecule ZT targeting *Taq* polymerase was also inserted in the same HI-loop but at a different position. The engineered fiber with ZT-ZH could bind to both targets, while the one with ZH-ZT could not (Myhre *et al.*, 2009).

Various Affibody molecules have been demonstrated to block relevant protein interaction. For example, an ABD-fused Affibody specific for CD28 could inhibit reaction between CD28- CD80. CD28 plays an important role in co-stimulatory signaling in T-cells (Sandström *et al.*, 2003). The Affibody molecules specific for CD25 were selected out from an Affibody phage library. The selected Affibody molecules were competitive for the same binding site with IL-2 known as the natural ligand of CD25. The overexpression of CD25 was found in organ rejections, some antoimmune diseases, and T-cell malignancies. Therefore, CD-binding Affibody could be potentially used as targeting agents (Grönwall *et al.*, 2008). Epidermal growth factor HER3 is considered as key biomarker in cancer. Two promising HER3-specific Affibody molecules were demonstrated to bind targets on three HER3 overexpressing cancer cell lines. Meanwhile, heregulin (HRG), regarded as natural ligand of HER3, was blocked by addition of Affibody molecules. *In vitro* experiment, these two Affibody molecules completely inhibited the HRG-induced cancer cell growth (Göstring *et al.*, 2012).

Furthermore, Affibody molecules own the ability to block soluble protein ligands. Affibody molecules targeting human TNF-α were selected an Affibody phage library. TNF-α is pro-inflammatory cytokine which cause fever, inflammation, and tis-

sue destruction. Twelve sequences of Affibody molecules were gained after selection and sequencing. After protein characterization, three kinds of Affibody molecules were demonstrated with strongest binding. The Affibody binding site was overlapped with the one for TNF-α receptor. Therefore, the interaction between TNF-α and its receptor could be efficiently blocked (Jonsson *et al.*, 2010). Another research was focused on the amyloid-β (Aβ) peptides specific Affibody molecule in Alzheimer's disease. Two selected most promising Affibody variants were demonstrated to efficiently capture Aβ peptides spiked to plasma and serum samples. These Affibody molecules could be potentially used for therapeutic application (Grönwall *et al.*, 2006). Recently, a new Affibody molecule, $Z_{A\beta3}(12–58)$ specific for Aβ with sub-nanomolar affinity, was modified to lock the peptide in monomeric. It was indicated that engineered Affibody molecules are useful for study metal-binding and other properties of monomeric Aβ (Lindgren *et al.*, 2013).

2.4 Ephrin-B3

The largest known subfamily of receptor tyrosine kinases (RTKs) is Eph family (Brambilla and Klein, 1995). The corresponding ligands are called Ephrin. Ephrin are divided into two families based on the structure and function: One is Ephrin-A which is tethered to the cell surface by a glycosylphosphatidylinositol (GPI) linkage, while the other one is Ephrin-B which is transmembrane ligand (Flanagan and Vanderhaeghen, 1998). Generally, the Ephrin-A ligands activate the EphA receptors, while Ephrin-B ligands interact with the EphB receptors. However, Ephrin-A5 is reported to bind and activate EphB2 receptors (Himanen *et al.*, 2004), and another exception is that EphA4 can bind to both ephrin-A and ephrin-B (Kullander and Klein, 2002). In vitro, each Eph receptor binds multiple Ephrin ligands, and each ligand binds to multiple receptors (Pasquale, 1997). The contact-dependent interactions between Eph and their Ephrin lead to Eph autophosphorylation on tyrosine and activation of RTK activity. The interactions are involved in mediating and regulating cell migration and axon pathfinding (Flanagan and Vanderhaeghen, 1998).

Ephrin-B3 is an approximately 50kDa protein encoded by the EFNB3 gene, and it belongs to the Ephrin-B class. Ephrin-B3 is highly expressed in brain, and important in brain development and in its maintenance. It has been demonstrated to play key roles to regulate axon guidance at the ventral midline of the embryonic mouse spinal cord (Kadison *et al.*, 2006). Furthermore, Ephrin-B3 is likely involved in tumorigenesis (Fox and Kandpal, 2004). It was illustrated that ephrin-B3 expression and phosphorylation were associated with increasing tumor grade in in human glioma cells (Nakada *et al.*, 2006). Ephrin-B3 was described as a putative signaling molecule in the combined treatment of non-small cell lung carcinoma cells with PKC 412 and ionizing radiation. Increased cell death response was observed after inhibition of Ephrin-B3 expression (Stahl *et al.*, 2013).

3. MATERIALS AND METHODS

General: All PCR extensions were performed by GeneAmp® PCR System 9700 (Applied Biosystems) using Phusion DNA polymerase (Finnzymes) and oligonucleotide primers (Eurofins MWG Operon). PCR products were purified with QIAquick PC purification Kit (Qiagen). DNA restriction enzymes were the product from New England Biolabs. Different centrifuges Heraeus Multifuge 3SR (Thermo), and Avanti J-26XP (Beckman Coulter) were applied. DNA sequencing was performed by 3730XL DNA Analyzer (Life Technologies).

3.1 Phagemid Vector Engineering

3.1.1 Phagemid vectors

The phagemid vector pAffi-100-Tryp (Figure 9) was constructed from pAffi1 (Grönwall *et al.*, 2007). There was a full-length g3p (D1, D2, and D3) in pAffi-100-Tryp, while there was a truncated g3p (D3) in pAffi1. Also, there was a new element Trypsin site before the full-length g3p in pAffi-100-Tryp. The phagemid vector pAffi-100-Tryp was constructed in three steps.

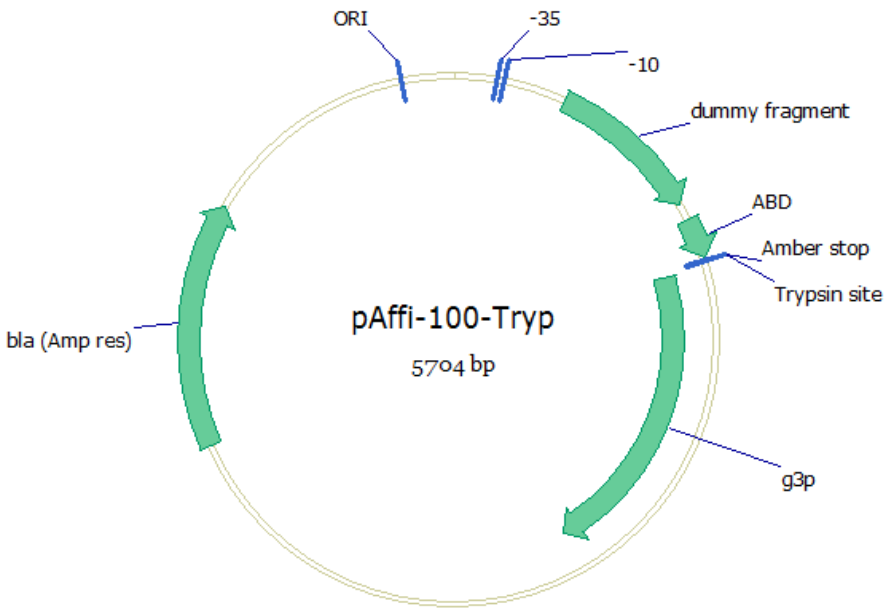

Figure 9. The phagemid vector pAffi-100-Tryp. Dummy fragment was kept in the position where Zlib should be inserted. There was an amber stop and a Trypsin site between ABD and g3p. This phagemid also contained Ampicillin resistance site.

In the first step, the gene fragments Helix3 and ABD following by introducing a trypsin cleavage site was obtained by using oligonucleotide primers DOLI1 (5'-GAAGCTT GCTAGCAGAAGCTAAAAG-3'; underline: *NheI* recognition site) and DOLI2 (5'-AGCCCCAGCACGGCGGGCCCCCTACCCGGGTACGTAAGGTAA-3'; underline: sequence complementary to DOLI3), and pAffi-1 was as template. The PCR reaction was performed with program consisting of initial denaturation at 98℃ for 30s, 30 cycles of denaturation 98℃/10s, annealing 61℃/30s and extension 72℃/15s, and final extension at 72℃ for 10 minutes. The sample was kept at 4℃ after completion. The other PCR reaction to get gene fragment full length g3p was carried out with the template pM13cp (Chastten *et al.*, 2006), as well as the primers DOLI3 (5'-GGGGCCCGCCGTGCTGGGGCTGAAACTGTTGA AAGTTGTTTTA-3'; underline: sequence complementary to DOLI2 for overlapping PCR) which also contained a trypsin site, and DOLI4 (5'-GAAAATTCATATGGTTTACAGCGC-3'; underline: *NdeI* recognition site). The PCR program consisted of initial denaturation at 98℃ for 30s, 30 cycles of denaturation 98℃/10s, annealing 59℃/30s, and extension 72℃/30s, and final extension at 72℃ for 10 minutes, and holding at 4℃ after completion.

Second, an overlap PCR was performed with the amplified PCR fragments Helix3 and ABD from pAffi1 and g3p from pM13cp, because there was a complementary part between DOLI2 and DOLI3. The outer primers DOLI1 and DOLI4 were added. The overlap-PCR program contained initial denaturation at 98℃ for 30s, 30 cycles of denaturation 98℃/10s, annealing 61℃/30s and extension 72℃/30s, and final extension at 72℃ for 10 minutes, and holding at 4℃ after completion. The PCR product was an overlapped fragment consisted of Helix3and ABD, Trypsin site, and g3p. This PCR fragment was purified via gel extraction. 15µl PCR product was mixed with 3µl 6X Loading Dye, and loaded in the well of 0.75% agarose gel stained with Ethidium Bromide. The electrophoresis was set for 25 minutes with 120V. The band of overlapped fragment was cut and purified with QIAquick Gel Extraction Kit (Qiagen).

In the third step, the overlapped fragment was inserted between the *NheI* and *NdeI* sites of pAffi1. Approximately 180ng of overlapped fragment was digested in 40µl volume reaction which included 10 units *NheI-HF®*, 10 units *NdeI*, 1X BSA, and 1X NEBuffer 4. The mixture was incubated at 37˚C for 1 hour, and deactivated at 80˚C for 20 minutes. There was approximately 6µg pAffi1 vector digested in 50µl volume reaction with 40 units *NheI-HF®*, 40 units *NdeI*, 1X BSA, and 1X NEBuffer 4. The digestion was performed at 37˚C for 2 hours, and the inactivation was processed at 80 ˚C for 20 minutes. Both of the digestion products were verified by electrophoresis on 1% agarose gel stained with Ethidium Bromide. 2µl of the PCR product was mixed with 2µl 2X Loading Dye, and loaded in the well. The electrophoresis was set for 25 minutes with 150V. The digested pAffi1 vector was purified by gel extraction. 50µl of digested pAffi1 vector was mixed with 10µl 6X Loading Dye. The mixture was loaded in the 0.75% agarose gel stained with Ethidium Bromide. The electrophoresis was set for 25 minutes with 130V. The band of digested pAffi1 vector was cut and purified with QI-

Aquick Gel Extraction Kit (Qiagen). The digested overlapped fragment was purified by QIAquick PCR Purification Kit (Qiagen).

Both of the purified digestion products were ligated in a 30µl volume reaction including approximately 50ng digested pAffi1 vector, approximately 80ng digested overlapped fragment, 1X T4 DNA Ligase Buffer(New England Biolabs), and 300 units T4 DNA Ligase (New England Biolabs). The mixture was incubated at RT for 1 hour. The ligation product was named as pAffi-100-Tryp.

3.1.2 DNA sequence analysis

In order to verify the sequence correction of phagemid pAffi-100-Tryp, it was transformed in to *E.coli* strain *Top 10* via heat shock. 2µl of ligation product was mixed with 8µl of 5X KCM buffer (500mM KCl, 150mM $CaCl_2$, and 250mM $MgCl_2$). After 3 minutes incubation on ice, the mixture was added into 10µl of thawed *Top 10* competent cells. Then, it was left on ice for 20 minutes, and moved back to RT for 10 minutes. The mixture was taken back on ice for another 3 minutes. Following adding 200µl of TSB+Y medium, the mixture was rotated e-o-e for 1 hour at 37 ℃, and spread on agar plate with Amp 100µl/ml. The plates were placed in 37℃ overnight.

8 colonies were picked for sequencing. These colonies were diluted with Milli-Q, and served as template for PA-PCR reaction. Primers DOLI4 and E' (5'-GCGCAACACFATGAAG-3') were used. The PA-PCR reaction was performed in 25µl reaction volume including 0.2µl *Taq* DNA polymerase, 1X *Taq* buffer, 0.1µM forward primer E', 0.1µM reverse primer DOLI4, and 100µM dNTP. The program contained initial denaturation at 94℃ for 5 minutes, 35 cycles of denaturation 94℃/40s, annealing 59℃/40s and extension 72℃/1min40s, and final extension at 72℃ for 8 minutes, and holding at 4℃ after completion. The PCR product verified by electrophoresis on a 1% agarose gel stained with Ethidium Bromide. 2µl of the PCR product was mixed with 2µl 2X Loading Dye, and loaded in the well. The electrophoresis was set for 25 minutes with 150V. The PCR fragments with correct length were saved for Cy-seq PCR. Cy-seq PCR was performed in 10µl reaction volume including 2 µl PA-PCR product as template, 0.5µl Big Dye, 1X CS buffer, and 0.5µM primer X (X stood for one of the five primers DOLI1, DOLI2, DOLI3, DOLI4, and E' which were used for Cy-seq PCR reaction separately). The program was set with initial denaturation at 95℃ for 30s, 35 cycles of denaturation 96℃/10s, annealing 50℃/5s and extension 60℃/4min, and final extension at 60℃ for 4 minutes, and holding at 4℃ after completion.

Ethanol precipitatation

After Cy-seq PCR, the PCR products were handled with ethanol (EtOH) precipitate in a 96 well plate. In the first step, 25µl 95% EtOH and 1µ 3M CH_3COONa (pH=5.2) were mixed well with 10µl Cy-PCR product, and the mixtures was spun at 3200g and 4˚C for 30 minutes. The 96 well plate was inverted in order to remove all supernatant. Then,

75µl 75% EtOH was added, and the plate was centrifuged at 3200g and 4˚C for 30 minutes. After all supernatant removed, the plate was kept in dark place for drying out. At last, 20µl Milli-Q was added, and the plate was sent for sequencing.

The colonies with correct sequence were picked out, and inoculated in 10ml TSB+Y medium with 100µg/ml Amp and cultivated at 37℃ overnight. The cells were harvested for plasmid extraction by QIAprep ® Spin Miniprep Kit (Qiagen).

3.2 Comparative Test Selections with Low pH acid and Trypsin elution

3.2.1 Preparation of phagemid pAffi-100-Tryp-Z_{wt} and pAffi-100-Tryp-$Z_{Her2:342}$

In order to test the new system, a comparative test was applied. The test selections were performed with the phage particle mixtures only containing Z_{WT} and $Z_{Her2:342}$. The phagemid pAffi-100-Tryp-Z_{WT} and pAffi-100-Tryp-$Z_{Her2:342}$ were prepared from phagemid pAffi-100-Tryp where the dummy fragment was replaced by gene fragment coding Helix1 and Helix2 which served as the randomization part of the library. Both of the DNA fragment (Z_{WT}) coding Helix1 and Helix2 of Z_{WT} and DNA fragment ($Z_{Her2:342}$) was amplified separately.

The PCR product of $Z_{Her2:342}$ was purified by gel extraction. 35µl PCR product was mixed with 7 µl 6X Loading Dye, and loaded in the well of 0.75% agarose gel stained with Ethidium Bromide. The electrophoresis was set for 25 minutes with 130V. The band of $Z_{Her2:342}$ was cut and purified with QIAquick Gel Extraction Kit (Qiagen). The PCR product of Z_{WT} was purified by QIAquick PCR purification kit (Qiagen).

Both of the phagemid pAffi-100-Tryp and insert DNA fragment coding Helix1 and Helix2 of Z_{WT} and $Z_{Her2:342}$ were digested with restriction enzyme *XhoI* and *NheI-HF*®.

Approximately 7.8ng phagemid pAffi-100-Tryp vector was digested in 40µl volume digestion reaction with 20 units *NheI-HF*®, 20 units *XhoI*, 1X BSA, and 1X NEBuffer 4. The digestion was performed at 37˚C for 1 hour, and the inactivation was processed at 60˚C for 20 minutes. The digested pAffi-100-Tryp vector was purified by gel extraction. 30µl of digested phagemid pAffi-100-Tryp vector was mixed with 7µl 6X Loading Dye. The mixture was loaded in the 0.75% agarose gel stained with Ethidium Bromide. The electrophoresis was set for 25 minutes with 130 V. The band of digested pAffi-100-Tryp vector was collected and purified with QIAquick Gel Extraction Kit (Qiagen).

Ligation reactions were performed for each insert pAffi-100-Z_{WT}-Tryp and pAffi-100-$Z_{Her2:342}$-Tryp. The ligation for insert Z_{WT} was prepared in 20µl volume reaction contains approximately 38ng digested DNA fragment Z_{WT}, approximately 52ng digested pAffi-100-Tryp vector, 1X T4 DNA ligase buffer, and 200 units T4 DNA ligase;

while the ligation for insert $Z_{Her2:342}$-Tryp was also made in 20µl volume reaction which includes approximately 45ng digested DNA fragment $Z_{Her2:342}$-Tryp, approximately 52ng digested pAffi-100-Tryp vector, 1X T4 DNA ligase buffer, and 200 units T4 DNA ligase. All of the ligations were incubated at RT for 1 hour. The ligation products were called pAffi-100-Z_{WT}-Tryp and pAffi-100-$Z_{Her2:342}$-Tryp. The ligation products were used for heat shock transformation with *E. coli* strain *Top 10* cell. After transformation, the cells were spread on agar plate with 100µg/ml Amp and left at 37℃ overnight. 24 colonies were picked up from overnight culture of Z_{WT} and $Z_{Her2:342}$ for sequencing. PA-PCR and Cy-seq PCR were performed as mentioned in Section 2.1.2. The colonies with correct sequence were saved for phage particle preparation.

3.2.2 Helper phage preparation

ER2738 cell (Lucigen) was cultivated in 50ml TSB medium with 10µg/ml Tet. The flask was shaken at 37℃ with 120 rpm. When the value of O.D. 600 reached 0.5-0.8, 10µl of helper phage KM13 (1.0×10^{11} pfu/ml, Creative Biolabs) was added. The flask was incubated at 37˚C without shaking, which was followed by half hour slowly shaking. Then, the mixture was transfer to 500ml TSB+Y medium with 50µg/ml Kanamycin and 10µg/ml Tet, which was shaken at 30℃ with 100 rpm about 16-18 hours.

PEG 6000/NaCl precipitation

The overnight cultured helper phage KM13 was centrifuged at 3000g for 20 minutes at 4℃. The supernatant was transfer to a new tube for PEG/NaCl (20% (w/v) PEG6000, and 2.5M NaCl) precipitation. After 1/4Vol PEG/NaCl added, the mixture was incubated on ice for 1 h. Then, it was centrifuged at 10500 g for 40 minutes at 4℃. The supernatant was poured off, and the phage pellet was resuspended in 1/20Vol 1X PBS buffer (154mM NaCl, 8.1mM Na_2HPO_4, and 1.9mM NaH_2PO_4*H_2O). The sample was removed into SS-34 tubes, and 1/4Vol PEG/NaCl was added. After 45 minutes incubation on ice, the sample was centrifuged at 12000g for 40 minutes at 4℃. The supernatant was poured off, and the pellet was resuspended in 2ml 1X PBS buffer. With another centrifuge at 13000g for 1min and filtration with 1.2µm and 0.45µm filter, the KM13 helper phage was collected. It was separately titrated with *E. coli* strain ER2738 on agar plates with Amp, plates with Km, and plates with Tet. The rest helper phage was stored at -20˚C after adding 1Vol 80% Glycerol.

Phage titration

96-well plate was used in titration. In the 1st well (10^{-1}), 10µl of helper phage KM13 was diluted with 90µl 1X PBS buffer. 10 µl of the mixture in the 1st well was taken out and diluted with 90µl 1X PBS buffer in the 2nd well (10^{-2}). The dilution series was made till the 10th well (10^{-10}). 100µl of ER2738 with O.D. between 0.5-0.8 was added to each

well (Table 1). After 5 minutes incubation, 10µl the mixtures from 5^{th} (10^{-5}) to 10^{th} (10^{-10}) were stretched on agar plate with 10µl/ml Ampicillin or agar plate with 10µg/ml Tetracycline. After 15 minutes incubation, 10µl the mixtures from 5^{th} (10^{-5}) to 10^{th} (10^{-10}) were stretched on agar plate with 10µl/ml Kanamycin. The plates were put in the 37˚C room. The number of colonies in each 10µl portion was counted for calculating the titer according to the Table 2 shown below.

Table 1. The related values for 10-fold serial dilutions. 100µl of *E. coli* strain *ER2738* was added after the serial dilution.

Well	10^{-1}	10^{-2}	…	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Phage	10µl (Stock)	10µl (10^{-1})	…	10µl (10^{-4})	10µl (10^{-5})	10µl (10^{-6})	10µl (10^{-7})	10µl (10^{-8})	10µl (10^{-9})
PBS	90µl	90µl	…	90µl	90µl	90µl	90µl	90µl	90µl
+									
E.coli	100µl	100µl	…	100µl	100µl	100µl	100µl	100µl	100µl

Table 2. Calculation of titer according to the plaque numbers in each 10µl portion. The plaques numbers were counted after overnight cultivation at 37˚C. The titer (pfu/ml) was calculated with the plaque number in 10µl volume portion multiply the corresponding coefficient for the dilution.

Dilution Well	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Volume for titration	10µl	10µl	10µl	10µl	10µl	10µl
Corresponding volume in stock	5×10^{-5}µl	5×10^{-6}µl	5×10^{-7}µl	5×10^{-8}µl	5×10^{-9}µl	5×10^{-10}µl
Coefficient	2×10^{7}	2×10^{8}	2×10^{9}	2×10^{10}	2×10^{11}	2×10^{12}

3.2.3 Phage particle preparation

The pAffi-100-Z_{WT}-Tryp phage stock and pAffi-100-$Z_{Her2:342}$-Tryp phage stocks were prepared separately. For the phage produce, the selected colony with pAffi-100-Z_{WT}-Tryp or pAffi-100-$Z_{Her2:342}$-Tryp vector was inoculated and cultivated in TSB+Y medium with 100 µg/ml Amp, 10µg/ml Tet. The overnight culture was re-inoculated into 100 ml TSB medium with 2% (w/v) Glucose, 100 µg/ml Amp, 10µg/ml Tet. The initial O.D.600 value was below 0.1, and the flasks were shaken at 37℃ with 120rpm. When the O.D. reached 0.5-0.8, 10 fold excess of helper phage KM13 was added. The culture was left in 37℃ without shaking for 30 minutes, centrifuged with 3500g for 10 minutes. The cell pellets were resuspended in 100 ml TSB+Y medium with 100µg/ml Amp, 25µg/ml Km, 10µg/ml Tet, and 0.1mM IPTG. The cells were cultivated at 30℃ with 100 rpm for overnight.

In the second day, the overnight culture was centrifuged at 3500g for 15 minutes. The supernatant was transferred for PEG/NaCl precipitation. After adding equal volume 85% Glycerol, it was saved at -20℃. Also, titration is performed with *ER2738* on agar plates with 100µg/ml Amp, 25µg/ml Km, 10µg/ml Tet.

3.2.4 Target protein ENBREL® preparation

Soluble tumor necrosis factor receptor (p75) fusion protein/Fc Chimera (ENBREL®) was used as target in the comparative selection. Affibody Z_{WT} was specifically bound to Fc part of ENBREL®, while affibody $Z_{Her2:342}$ acted as control variant. Also, it served as target in the pre-selection for the Naïve Affibody library. 200µl ENBREL® (500µg/ml) was biotinylated using EZ-Link TM Sulfo-NHS-LC-biotin (Thermo Fisher Scientific). A 20 molar fold excess of biotin was added to the protein solution and incubated at RT for 30 minutes. The mixture was injected into Slide-A-Lyzer ® 3.5k Dialysis cassettes (Thermo Scientific), and incubated in 1X PBS buffer with rotation at 4℃ overnight. The biotinylated target protein immobilized on Dynabeads ® M-280 Streptavidin (Invitrogen) was tested. 75 µl Dynabeads ® M-280 Streptavidin was washed with 1X PBST, and divided into 3 Eppendorf tubes which marked with Tube 1, Tube 2 and Tube 3. 3µl biotin-p75 reagent and 172µl 1X PBST were added to Tube 1. Tube 1 was rotated at RT for 30 minutes. Then, the liquid without beads in Tube 1 was removed into Tube 2, and Tube 2 was rotated in RT for 30 minutes. The liquid was then removed to Tube 3, and rotated for another 30 minutes. At last, the liquid was transferred to a new Eppendorf tube Tube 4. All the four tubes were saved for the SDS-PAGE. The SDS-PAGE was performed with the Mini-PROTEAN® TGX™ Precast Gels (BIO-RAD) at 4˚C for 45min with 140V.

3.2.5 Selections with low pH acid and Trypsin elution

During the selection, all the Eppendorf tubes were blocked with 1X PBST buffer (1X PBS buffer with 0.1% (v/v) Tween) and 5% BSA. Beads were split into 100µl in each tube, and washed with 1X PBST 3 times before using as well. The phage particles of Z_{WT} and $Z_{Her2:342}$ were mixed as the ratio 1:10000, and the total volume was 1ml. The phage mixture was added to the beads and e-o-e rotated for 30 minutes at RT. The supernatant was transfer to a new tube. Then, 7.5µl biotinylated ENBREL® (500µg/ml) was added. After 2 hours e-o-e incubation at RT, anther 100µl beads were added to capture target protein and phage binders. The mixture was e-o-e incubated for 15 minutes at RT. The supernatant was discarded, while the left beads were washed 6 times with 1X PBST buffer. The washed beads were divided equally into 2 tubes. One was for Glycine-HCl elution, and the other was for Trypsin elution. In the Glycine-HCl elution, 500µl 50mM Glycine-HCl (pH2.2) was added for 10 minutes with e-o-e, then 450µl 1X PBS and 50µl 1M Tris-HCl (pH8.0) was added. In the Trypsin elution, the Trypsin stock solution (10mg/ml) was prepared in Trypsin Buffer (50mM Tris-HCl pH=7.5, and 1mM CaCl2), and stored at -20˚C. Trypsin solution with 1mg/ml was made from the stock before the elution. 200 µl Trypsin (1mg/ml) was added for 15 minutes with e-o-e, then 300µl 1X PBS buffer was added. 10 µl of elution product was taken out for titration on agar plates with 100µg/ml Amp. The rest was dropped to infect *ER2738* cell cultured in 50 ml TSB+Y medium when the O.D. 600 value was 0.5-0.8. After incubation at 37℃ without shaking for 30 minutes, the culture was centrifuged 3300g for 15 minutes. The medium was poured off, and the cell pellets were dissolved in 2ml TSB+Y medium and plated on TYE plate. The plates were left at 37 ℃ overnight.

3.3 Construction of Large Naïve Affibody Library in pAffi-100-Tryp

3.3.1 Preparation of library inserts and pAffi-100-Tryp vector

The complete sequence of the new library oligonucleotide Tri-121-mer (Ella Biotech) contains 162 bases, of which 121 bases comprised the Helix1 and Helix2 of Z domain. The library was based on the WT scaffold. The codons for Cys and Pro were avoided, and all the other amino acids were distributed equally in the 13 random positions. Moreover, in Pos.31, only 60% of Ile, 10% of Asp, 10% of His, 10% of Lys, and 10% of Tyr were included. The sequence of Tri-121-mer was listed in Appendix.

In order to amplify the library oligonucleotide Tri-121-mer, the Forward primer (5'-GATGAAGCCCTCGAGGTAGACAACAAATTCAACAAAGAA-3; underline: the overlap with Tri-121-mer) and Reverse primer (5'-TTAGCTTCTGCTAGCAG CAAGTTAGCGCTTTGGCTTGGGTCATC -3'; underline: the overlap with Tri-121-mer) were ordered together. The Tri-121-mer oligo was dissolved in 150µl 1X TE buff-

er, and the stock concentration was 923ng/µl. Before used for PCR, the stock was diluted 42 times to get the concentration 22ng/µl. In each 50µl volume reaction, there was approximately 22ng template Tri-121-mer oligo, 0.5µM Forward primer, 0.5µM Reverse primer, 200 µM dNTPs, 1X Phusion HF Buffer (Biolabs® NEW ENGLAND), and 1 unit Phusion DNA Polymerase (Biolabs® NEW ENGLAND). Totally, there were 96 reactions. The program was set as the initial denaturation at 98℃ for 30s, 30 cycles of denaturation 98℃/10s, annealing 60℃/30s and extension 72℃/30s, and final extension at 72℃ for 10 minutes, and holding at 4℃ after completion. PCR product was pooled together and purified. Totally, there was approximately 6.5mg of Tri-121-mer fragment. The phagemid vectors were prepared from the cells containing pAffi-100-Tryp by NucleoBond ® Xtra Midi/Maxi.

Both of the vector pAffi-100-Tryp and extension product Tri-121-mer were digested with *XhoI* and *NheI* at 37 ℃. After 4h incubation for pAffi-100-Tryp and 3.5 h for Tri-121-mer, the reaction mixtures were deactivated at 65℃ for 30 minutes. Each cleavage was purified by gel extraction (QIAquick Gel Extraction Kit). 8.0 µg of Tri-121-mer insert and 17.9µg of pAffi-100-Tryp vector were subjected to ligation using T4 DNA ligase in 1.6ml reaction volume. The mixture was incubated at RT for 4 hours, and at 4℃ for 3 days.

The ligation product was extracted by Phenol/Chloroform. Equal volume as ligation reaction of Phenol:Chloroform:isoamylalcohol (25:24:1) was added. After 2 minutes vortex, the sample was centrifuged at 10,000 g for 5 minutes at 4℃. The aqueous (upper) layer was transferred to a new tube. Equal volume of Phenol:Chloroform: isoamylalcohol was added. Another 2 minutes vortex was applied, which followed by 5 minutes centrifuge at 10000g at 4℃. The aqueous layer was collected for ethanol precipitation. 1/10Vol of 3M sodium acetate (pH5.5) and 2.5Vol of ice cold 95% ethanol were added. After a brief vortex, the mixture was incubated at -20℃ for 1 hour and centrifuged at 21500g for 1 hour at 4℃. The supernatant was removed, and 1ml ice-cold 70% ethanol was added. The mixture was spun briefly. The decant supernatant moved, and air dry was applied to the pellet. At last, the pellet was dissolved in 140µl EB buffer.

The concentration of purified ligation was determinate by the transformation efficiency where 1µl ligation was electro-transformed with 25µl *E.coli* strain *ER2738* (Lucigen). 1µl ligation product was mixed with 25µl E.coli strain ER2738 and added into the Gene Pulser/MicroPulser Cuvette (BIO-RAD) with 0.2cm gap. The transformation was performed with MicroPulser^{TM} electroporator (BIO-RAD) with 1.8kV and 1 pulse. After the electro-transformation, the mixture was immediately removed back to Eppendorf tube, and 974µl pre-warmed SOC medium (TSB+Y medium with 5% (w/v) glucose, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$) was added. The tube was rotated at 37˚C for 1h. 200µl of the culture was transferred to the 1st tube (10^{-1} tube) with 800 µl TSB+Y medium for a tenfold serial dilution. After totally mixed, 200 µl of culture from 10^{-1} tube was transferred to the 2nd tube (10^{-2} tube). The 10-fold serial dilution continued till the 10^{-5} tube. 200 µl of culture was taken out from 10^{-2} to 10^{-5} tubes

for plating on agar plates with 100µg/ml Amp and 10µg/ml Tet. The plates were kept in 37˚C overnight. The colonies were counted in the second day. The transformation efficiency was approximately 1.28×10^8 colonies per shoot.

3.3.2 Electro-transformation with *E. coli* strain *ER2738*

The ligation products were electro-transformed into *E. coli* strain *ER2738* (Lucigen). 2µl of extract was mixed with 25µl of *E. coli*, and added into the Gene Pulser/MicroPulser Cuvette (BIO-RAD) with 0.1cm gap. The instrument MicroPulser^{TM} electroporator (BIO-RAD) was used, and the preset conditions were set as 1.8kV with 1 pulse. After each electro-transformation, the mixture was immediately removed back to Eppendorf tube, and 875µl pre-warmed Recovery Medium (Lucigen) was added. Then, the cells and medium were transferred to a 15ml Falcon tube. Every 7 or 8 transformations were pooled together. Total 31 electro-transformations were made, and mixed into 4 pools for overnight cultivation. Each pool mixture was left in 1L TSB+Y medium with 100 µg/ml Amp and 10µg/ml Tet.

Titration was done after 1 hour incubation and before pouring into big overnight cultivation. 100µl culture was taken out from each flask, and a 10-fold serial dilution was applied. In the 1st tube (10^{-1} tube), 100µl culture was mixed with 900µl TSB+Y medium. After well mixed, 100µl culture from 10^{-1} tube was transferred to the 2nd tube (10^{-2} tube). The 10-fold serial dilution continued till the 10^{-7} tube. 100 µl of culture was taken out from 10^{-3} to 10^{-7} tubes for plating on agar plates with 100µg/ml Amp. The rest culture in 10^{-1} to 10^{-3} tubes was poured back to the flasks. The plates were kept in 37˚C overnight. The flasks were shaken at 37℃ with 120rpm. After overnight cultivation, O.D. 600 of each pool was measured in order to calculate the copy of each pool. Four pools were mixed together with the same copy number. The cells were harvested by centrifuge 7 minutes at 4000g. Then, the pellets were dissolved in 40% Glycerol. The library cell pellets were split equally into 4ml in 15ml Falcon tube, and total was 8 tubes. These Falcon tubes were stored at -70 ℃.

3.3.3 Helper phage and phage particle preparation

More helper phage particle was prepared. The steps were similar to the one mentioned in the section 3.2.3. The cultivation (TSB+Y medium with 25µg/ml Km, and 10µg/ml Tet) volume increased to 2L. 4ml of library cell was inoculated into 6L TSB medium with 2% (w/v) Glucose, 100µg/ml Amp, 10µg/ml Tet. The initial O.D.600 was below 0.1, and the flasks were shaken at 37℃ with 120rpm. When the O.D. reached 0.5-0.8, 10 fold excess of helper phage KM13 (6.9×10^{12} pfu/ml) was added. The culture was left in 37℃ without shaking for 30 minutes, and then was centrifuged with 3500g for 10 minutes. The cell pellets were resuspended in 6L TSB+Y medium with 100µg/ml Amp, 25µg/ml Km, 10µg/ml Tet, and 0.1mM IPTG. The cells were cultivated at 30℃ with 100rpm for overnight. The overnight culture was centrifuged at 3500g for 15 minutes to

harvest the phage. The supernatant was transferred for PEG/NaCl precipitation which was mentioned in Section 3.2.2. The collected phage particle was titrated with *E. coli ER2738* on agar plates with 100μg/ml Amp. The phage particles were stored in 4˚C.

3.3.4 Sequencing of cell library and phage library

96 colonies were sequenced from cell library and phage library to check the 18 amino acids distribution in randomization positions. For the cell library, 96 single colonies were randomly picked up from the titration plates. For the phage library, phage particle was added into *E. coli* strain *ER2738* cultivation when the O.D. value is between 0.5-0.8. After 30 minutes incubation at 37˚C without shaking, 1 ml of the mixture was taken out for series dilutions and spread on agar plates with 100μg/ml Amp. The plates were put at 37˚C overnight. One the second day, 96 single colonies were randomly picked up. PA-PCR was performed as Section 3.1.2. RIT27 and DOLI2 were chosen as forward and reverse primers. In the Cy-seq PCR, primers pAffi-100-For (5'-CACACAGGA AACAGCTATGACCATG-3') and ABD-Rev (5'-CTATCAGTGCTTTTACACCTTC AACAG-3') were used. The program was changed with initial denaturation at 98℃ for 30s, 30 cycles of denaturation 98℃/10s, annealing 62℃/30s, and extension 72℃/30s, and final extension at 72℃ for 10 minutes, and holding at 4℃ after completion. After ethanol precipitate, Cy-seq PCR product was dissolved in Milli-Q and sent for sequencing.

3.4 Selection to target protein rhEphrin-B3

3.4.1 Target protein rhEphrin-B3 Preparation

Recombinant Human Ephrin-B3/Fc Chimer (rhEhprin B3, R&D systems) was chosen as target in the Naïve Affibody library selection. 1ml rhEphrin-B3/Fc Chimera with the concentration 100μg/ml was biotinylated using EZ-Link TM Sulfo-NHS-LC-biotin (Thermo Fisher Scientific). A 20 molar fold excess of biotin was added to the protein solution. The mixture was injected into Slide-A-Lyzer® 2K Dialysis Cassettes (Thermo Scientific), and incubated in 1XPBS buffer with rotation at 4℃ overnight. The test for the amount of target immobilized on Dynabeads ® M-280 Streptavidin (Invitrogen) was prepared, which was determined by SDS-PAGE.

3.4.2 Selection with low pH acid and Trypsin elution

The selection was performed essentially as described in Section 3.2.5 with some modifications. A pre-selection to ENBREL® was made before the selection to target protein rhEhrin-B3, and there were four round selections.

In Round 1, two tubes with 1 ml/tube naïve phage library (1.8×10^{10} cfu/ml) were used for selections. In the pre-selection, 15µl biotinylated ENBREL® (500µg/ml) was added in each tube. After 1h e-o-e incubation at RT, 100µl beads were added to capture ENBREL® and phage binders. The mixture was e-o-e incubated for 15 minutes at RT. The supernatant was removed and equally divided into new blocked Eppendoff tube marked with Tube 1 and Tube 2. Then, 50µl bio-Ephrin-B3 was added into Tube 1, while 50µl bio-Ephrin-B3 and 10µl Ephrin-A2 were added into Tube 2. The mixtures were incubated at RT for 2h, and transferred to new blocked tubes with 125µl pre-wahsed beads. The mixture was e-o-e rotated for 15 minutes at RT. The supernatant was discarded, and the beads were washed 3 times with 1X PBST. Tube 1 was divided into Track 1 and Track 2, while Tube 2 was divided into Track 3 and Track 4. Track 1 and Track 3 were eluted with Glycine-HCl, while Track 2 and Track 4 were eluted with Trypsin. In the Glycine-HCl elution, 500 µl 50mM Glycine-HCl (pH2.2) was added for 10 minutes with e-o-e, then 450 µl 1X PBS buffer and 50µl 1M Tris-HCl (pH8.0) was added. In the Trypsin elution, 200 µl Trypsin (1mg/ml) was added for 15 minutes with e-o-e, then 800 µl 1X PBS buffer was added. 10 µl of elution product from each track was taken out for titration on Amp plates. The rest 990 µl was dropped to infect 200 ml *ER2738* cell when the O.D.600 value was around 0.5-0.8. After incubation at 37℃ without shaking for 30 minutes, the culture was centrifuged 3300g for 15 minutes. The medium was poured off, and the cell pellets were dissolved and plated on TYE plate. The plates were left at 37 ℃ overnight.

In the second day, the colonies were scraped off with TSB medium. A portion of the scraped cell suspensions was diluted to measure the O.D.600 value. For phage particle produce, the scraped cell suspensions were inoculated in 200ml TSB medium (2% (w/v) glucose, 100µg/ml Amp and 10µg/ml Tet), where the initial O.D600 value was 0.1. The flasks were shaken at 37˚C with 150 rpm. The following steps were the same as the section 3.2.3 to prepare the phage particles.

From Round 2 to Round 4, the final concentration of rhEphrin-B3 was decreased which was 50nM in Round 2, 10nM in Round3, and 5nM in Round4. The parameters for each selection were shown in Table 3. In the Round 4, the elution products were saved without infecting *E. coli* to produce phage particle.

After overnight cultivation of cells infected by the elute from Round 4 on agar plates with 100µg/ml Amp, 48 colonies were randomly picked from each track for sequencing. The steps and programs were the same with the ones for sequencing the library which were mentioned in Section 3.3.4. The following work including analysis of the sequencing results and Affibody biding study was performed by Feifan Yu.

Table 3. An overview of the selection parameters. There were four tracks performed. Track 1 and Track 3 were eluted by Glycine (pH2.2), and Track 2 and Track 4 were eluted by Trypsin. The concentration of rhEphrin-B3 was decreased, while wash time was increased.

	Pre-selection (ENBREL®)	Selection (rhEphrin-B3)	Elution
Round 1	100 nM	100 nM 3 × washes	Glycine (pH 2.2) 10 minutes
			Trypsin 25 minutes
Round 2	100 nM	50 nM 5 × washes	Glycine (pH 2.2) 10 minutes
			Trypsin 25 minutes
Round 3	50 nM	10 nM 8 × washes	Glycine (pH 2.2) 10 minutes
			Trypsin 25 minutes
Round 4	33 nM	1 nM 10 × washes	Glycine (pH 2.2) 10 minutes
			Trypsin 25 minutes

4. RESULTS

4.1 Phage display system design

Sequencing of g3p in phagemid pAffi-100-Tryp

The phagemid vector called pAffi-100-Tryp was constructed from pAffi-1. The full-length g3p took the place of truncated g3p (D3), and a trypsin-sensitive linker was introduced between library member and p3 in the resulting vector. The sequencing results of g3p were checked and compared the III attachment protein [*Enterobacteria phage M13*] in NCBI (Shown in Appendix) (http://www.ncbi.nlm.nih.gov/gene/?term=m13+pIII). However, there were some mutations in g3p which listed in Table 4. Totally, there were eight mutation positions in DNA level, which influenced seven amino acids. Four out of seven were synonymous mutations, which did not make amino acid change. P11S was located in the N1 domain, P198L was in the N2 domain, and S360G was in the CT domain. However, the mutations did not prevent phage particles to infect *E.coli* cells.

Table 4. DNA mutations and amino acid change in the phagemid pAffi-100-Tryp

Position	10	11	35	198	199	360	368
G3p in NCBI	AAA→L	CCC→P	GGT→G	CCA→P	TTC→F	AGC→S	TCT→S
G3p sequenced	AAG→L	TCC→S	GGC→G	TTA→L	TTT→F	GGC→G	TCA→S

Comparative selection by low pH elution and Trypsin elution

In the comparative test selection, either Z_{WT} gene or a control variant $Z_{Her2:342}$ was inserted in the place of Dummy to get the phagemid pAffi-100-Z_{WT}-Tryp and pAffi-100-$Z_{Her2:342}$-Tryp. After the electro-transformation into *E. coli* strain *ER2738*, the cells harboring pAffi-100-Z_{WT}-Tryp and pAffi-100-$Z_{Her2:342}$-Tryp were gained. With the helper phage KM13, the phage particles with Z_{WT} and $Z_{Her2:342}$ type Affibody were produced. The titer for Z_{WT} phage particle on agar plate with 100μg/ml Ampicillin was 9.2×10^{10}cfu/ml, while it was 2.2×10^{10}cfu/ml for $Z_{Her3:242}$ phage particle.

The phage mixture was prepared by Z_{WT} phage particle and $Z_{Her2:342}$ phage particle as the ratio 1: 10000, and the amount of Z_{WT} phage particle was 2.0×10^{6} cfu. After the selection, the amount of phage particle from Glycine elution was 1.2×10^{5} cfu (6%), while it was 9.2×10^{4} cfu (4.6%) from Trypsin elution. 96 colonies from each track were

picked for sequencing. There were 84 readable sequences from Glycine elution and 96 readable sequences from Trypsin elution. All the sequences both in Glycine elution and Trypsin elution were Z_{WT}. It showed that the new phagemid pAffi-100-Tryp worked, and both low-pH acid and Trypsin were possible to elute the phage after the selection.

4.2 Protein preparation

Both of ENBREL® and rhEphrin-B3/Fc Chimera were biotinylated with biotin reagent in a molar excess of 20:1. The minimal amount of streptavidin coated beads possible to bind to 3.75µg ENBREL® and 5µg rhEphrin-B3/Fc Chimera was determined. With the binding assay, 100µl beads were needed for 3.75µg ENBREL®, while 125µl beads were needed for 5µg rhEphrin-B3/Fc Chimera

The results about the binding assay of rhEphrin-B3 were shown in Figure 10. There was no much difference between the molecular weight before and after dialysis. In the binding assay, there were 3 tubes each containing 25µl streptavidin coated beads used. The rhEphrin-B3 was only found in 1st tube. Therefore, all rhEphrin-B3 was able to bind to beads in the 1st tube, and none was transferred to the 2nd and 3rd one. In another word, 25µl beads were enough to bind 1µg of rhEphrin-B3.

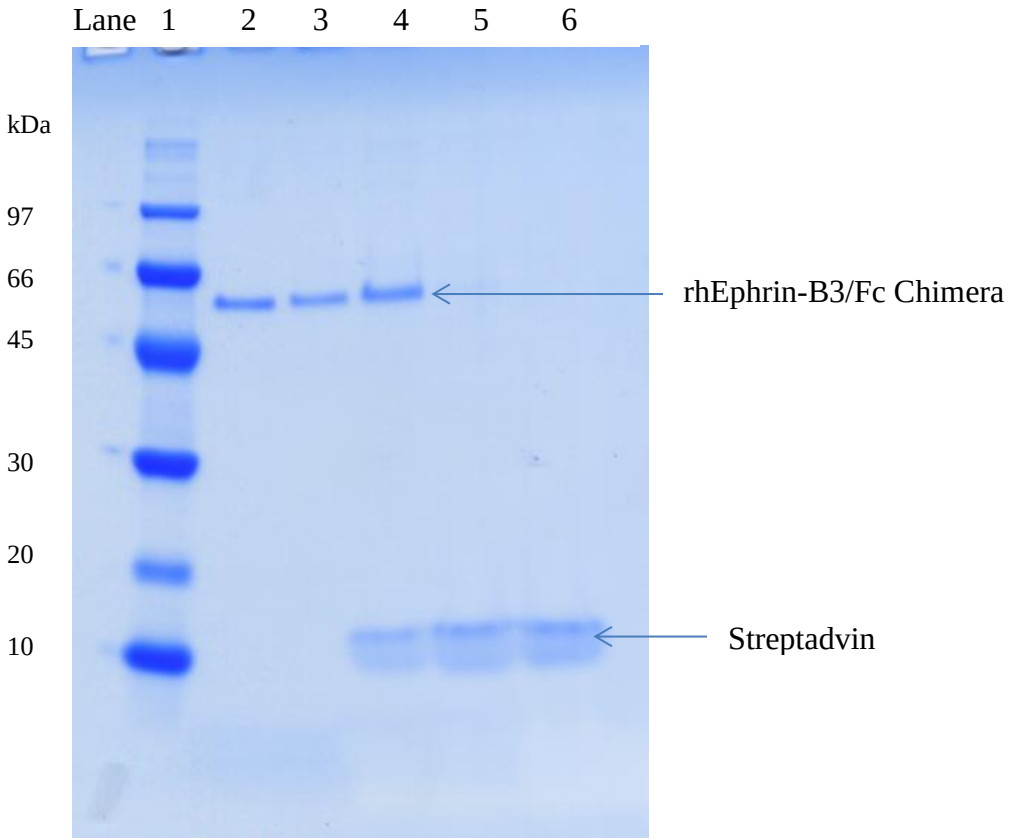

Figure 10. Binding assay with SDS-PAGE. From left to right, the sample in each lane was 1) Low molecular weight (LMW) ladder, 2) rhEphrin-B3 before dialysis, 3) rhEphrin-B3 after dialysis 4) 1st tube in binding assay 5) 2nd tube in biding assay. 6) 3rd tube in binding assay.

4.3 Cell library and phage library

The cell library was constructed by 31 electro-transformations. The size of cell library was 4.7×10^9 cfu. The cells were harvested and concentrated into 23.5ml, and the copy number was approximately 32 in 1ml. The sequencing results of library from 2×96 wells plates were summarized (Figure 11): 1) There were 140 readable sequences out of 192 samples; 2) 31 clones contained insertion or deletion, 1 clone contained mutation, and 1 clone contained Dummy. 3) 6 clones had Proline. 4) Therefore, the number of functional clones was 107, and the percentage of functional clones was 76.4%. The amino acid distribution in the randomization positions from the 107 functional clones was shown in Figure 6. In the Position 10, 11, 14, 24, 28, 32, Proline was found. The range of each amino acid percentage was from 1% to 15%, excluding in the Pos. 31. There was 61% Ile, 7% Lys, 10% Tyr, 11% His, and 12% Asp in the Pos. 31.

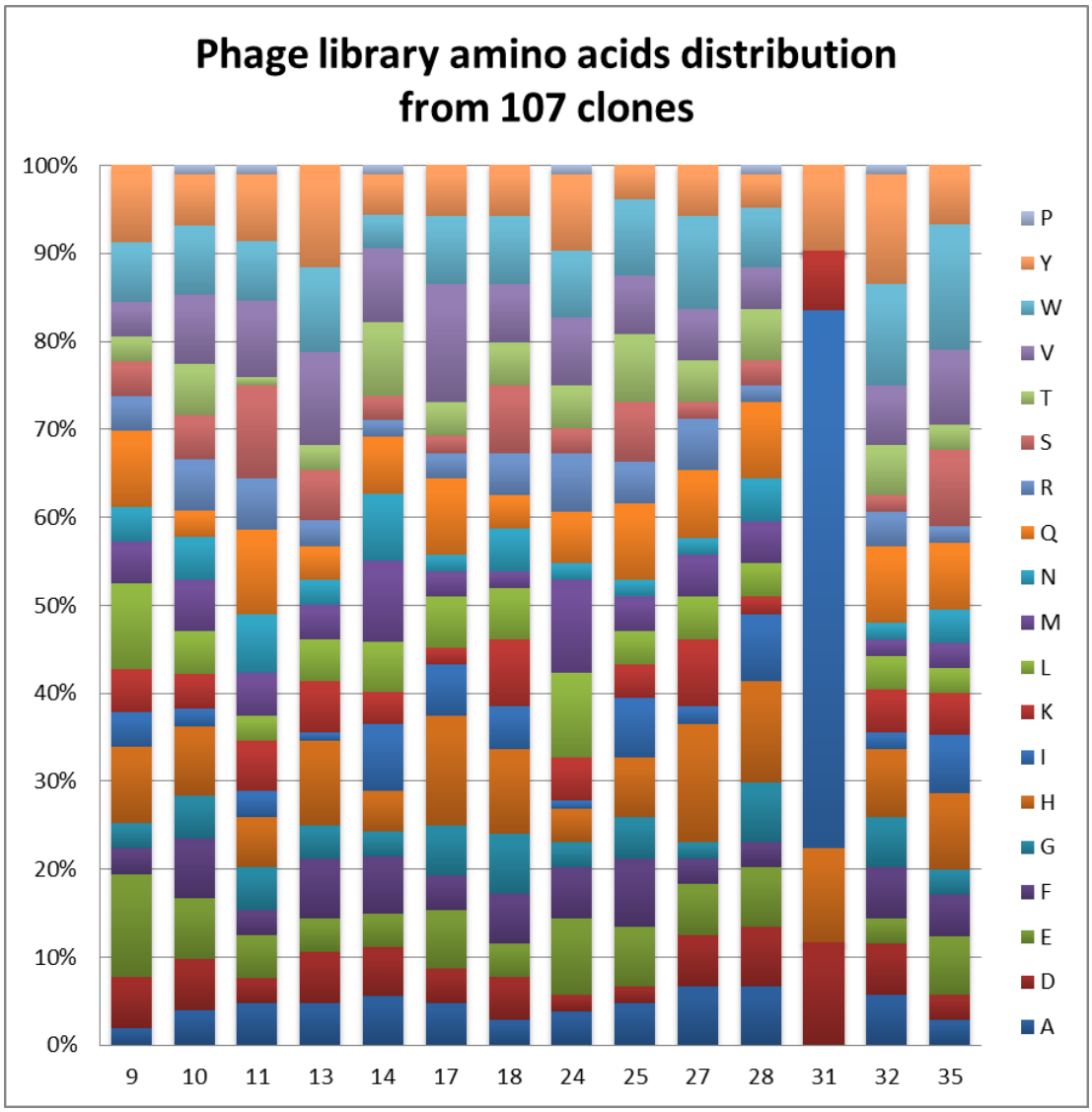

Figure 11. Amino acid distribution in 14 randomization from 107 functional clones. The distribution was shown in percentage. The theoretical value was about 5.56% for each amino acid in each position excluding Pos. 31.

4.4 Selection to rhEphrin-B3/Fc Chimera

The four round selections were performed with a pre-selection to ENBREL® in each round. In order to increase selection stringency, the number of washes was increased for each additional round and the target concentration was decreased. A figure about the enrichment from Round 2 to Round 4 was made (Figure7). There was obvious enrichment after Round 3 selection for Glycine (pH=2.2) elution, while it was Round 2 for Trypsin elution.

After four round selections, 48 clones were randomly picked from each track for candidates sequencing. The amino acids in the randomization positions in readable clones were given in the table 5.2. In all the four tracks, Z_{WT} was found after Round 4 selection. In Track 1, 2, and 3, clones harboring the phagemid expressing Z_{WT} occupied more than 64%. However, it was possible to get repeat clones in each track, which meant that the repeated clones might be potential binders to rhEphrin-B3. The sequencing results from each Track were shown in Table 5.

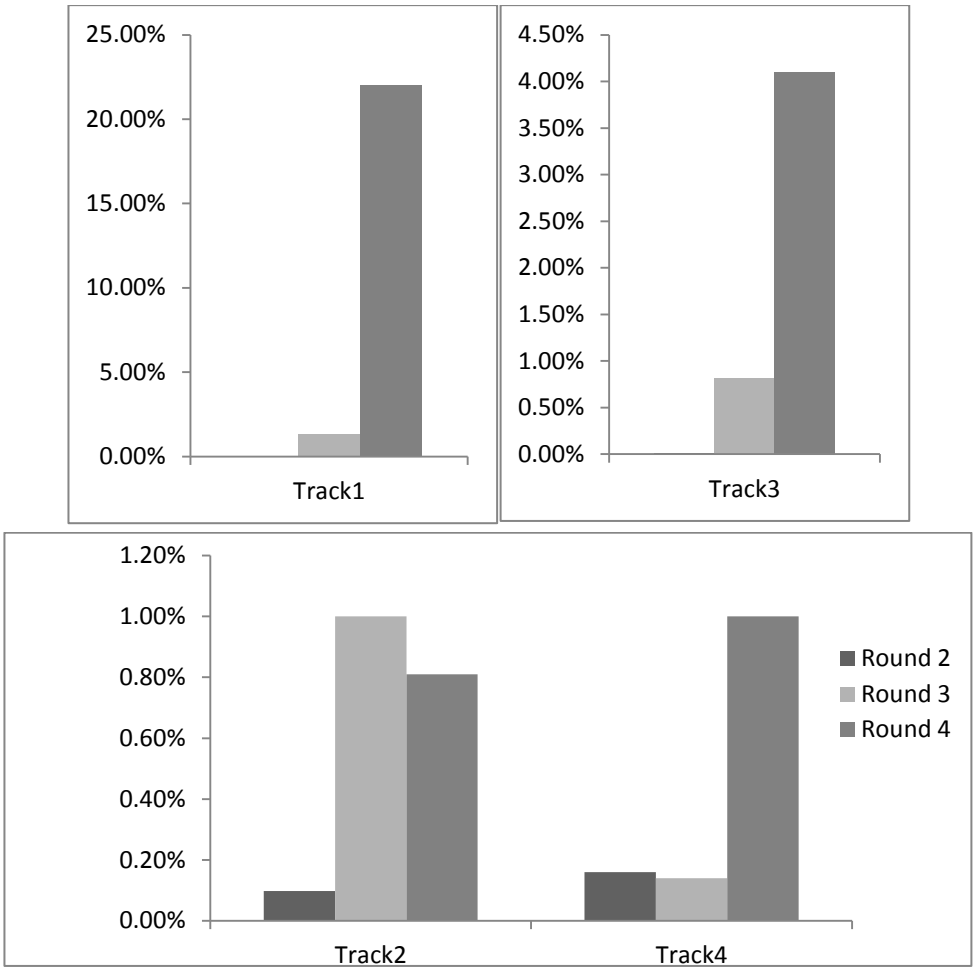

Figure 12. Phagemid enrichment from Round 2 to Round 4 in each Track. Track 1 and Track 3 were eluted by Glycine (pH2.2), while Track 2 and Track 4 were eluted by Trypsin.

4.5 Biacore

The binding properties of the selected Affibody were measured with Biacore. The clones with highest repeats in each track excluding Z_{WT} were chosen for Biacore measurements. The protein rhEphrin-B3/Fc Chimera and p75-Fc (ENBREL®) as a negative control were immobilized on chip. As shown in Figure 13, all the candidates were only able to bind to rhEphrin-B3/Fc, and none was able to bind to p75-Fc. Therefore, the binding sites of rhEphrin-B3/Fc were not Fc part, and the candidates were real binders to rhEphrin-B3. The candidate from Track 4 (T4) had the greatest response which was more than 50 resonance units (RU). The responses for candidate from Track 1 and candidate from Track 2 were quite similar, which were only about 10 RU. Moreover, the candidate from T4 had a very slow off rate.

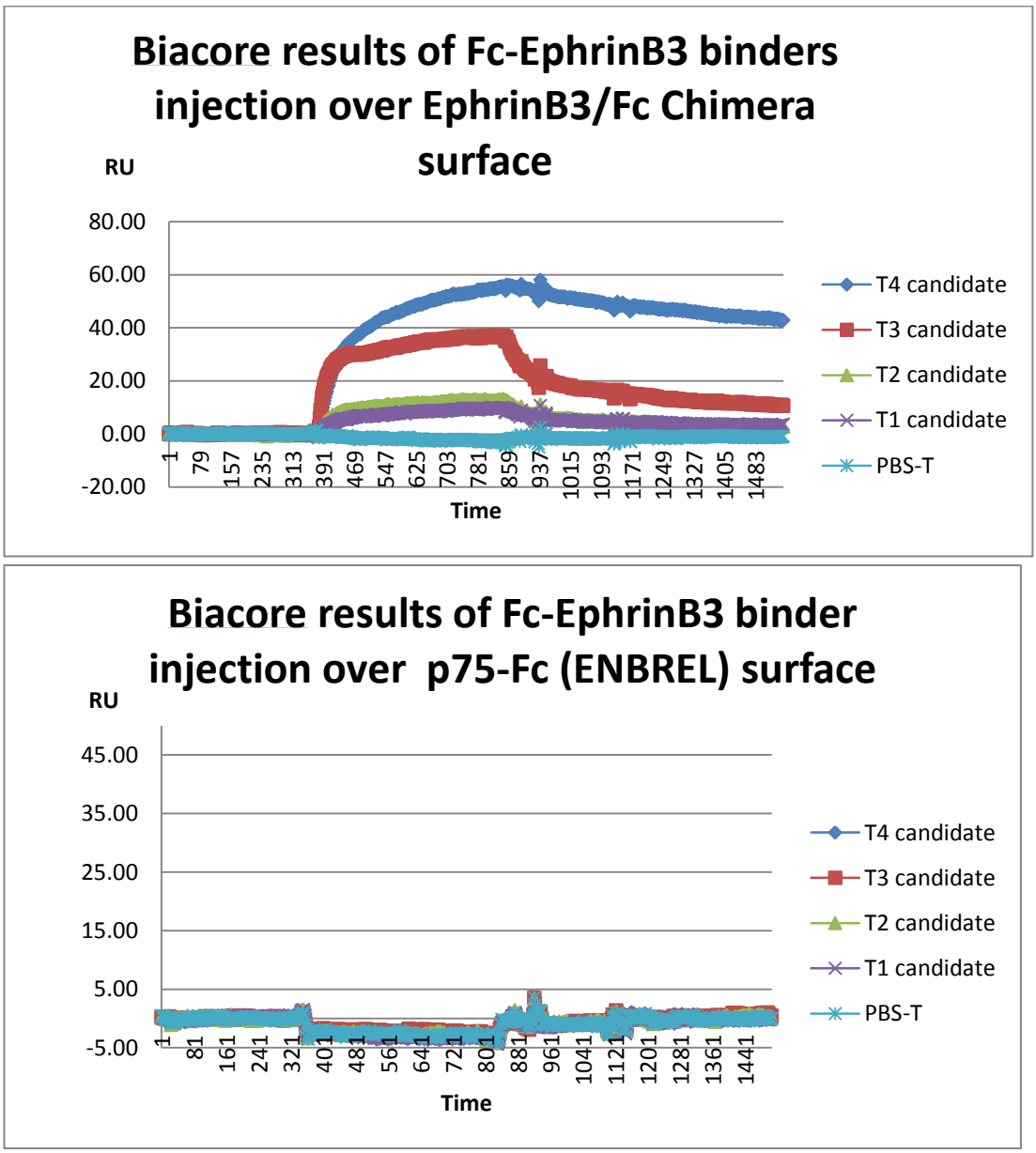

Figure 13. Biacore sensorgram. Four different Affibody molecules from selected clones were injected over the sensor chip immobilized with rhEphrin-B3/Fc Chimera and p75-Fc (ENBREL®).

Table .5 Sequencing results of picked clones in each track.

Position		10					20				30				40	50	
		↓					↓				↓				↓	↓	
Zwt	VDNKFNKE	QQN	A	FY	EI	LH	LPNLN	EE	Q	RN	AF	IQ	SL	K	DDPSQSANLLAEAKKLNDAQAPK		Repeat
Track 1	————————	QEV	–	LT	––	LD	—————	HE	–	MM	––	IS	––	W	————————————————————		8
+ 35 Zwt	————————	MDQ	–	MD	––	MK	—————	HE	–	VM	––	IH	––	W	————————————————————		2
	————————	REI	–	WW	––	VQ	—————	HA	–	VQ	––	IE	––	W	————————————————————		6
Track 3	————————	AHE	–	WW	––	VD	—————	ST	–	VA	––	IL	––	H	————————————————————		3
+ 24 Zwt	————————	WEM	–	MK	––	KE	—————	ST	–	HY	––	II	––	Y	————————————————————		1
	————————	FEA	–	FI	––	FG	—————	HD	–	EM	––	IA	––	W	————————————————————		1
	————————	WEF	–	YE	––	RL	—————	HE	–	AM	––	IK	––	W	————————————————————		1
	————————	HQM	–	YE	––	YH	—————	HM	–	MN	––	IH	––	W	————————————————————		1
	————————	VDA	–	FE	––	LG	—————	HE	–	VQ	––	IH	––	W	————————————————————		11
Track 2	————————	FEQ	–	YA	––	FT	—————	HI	–	EM	––	IH	––	W	————————————————————		2
+ 28 Zwt	————————	RDH	–	YN	––	SS	—————	HE	–	AM	––	IS	––	W	————————————————————		1
	————————	QEQ	–	YL	––	SS	—————	HG	–	VQ	––	IH	––	W	————————————————————		1
	————————	RDM	–	YL	––	LD	—————	HS	–	VY	––	IA	––	W	————————————————————		36
Track 4	————————	RQK	–	YY	––	MA	—————	HA	–	VI	––	IN	––	W	————————————————————		2
+ 3 Zwt	————————	FER	–	YL	––	FN	—————	HK	–	EM	––	IY	––	W	————————————————————		1
	————————	QKH	–	YD	––	YG	—————	HQ	–	VM	––	IT	––	W	————————————————————		1

5. DISCUSSION

5.1 Design of a phage library project

Generally, there are three stages for performing display selection from a library: 1) creating a library of (poly)peptide variants; 2)selection; and 3) analysis of the selected clones (Russel *et al.*, 2004). The flow diagram illustrating a phage display project is shown in Figure 14.

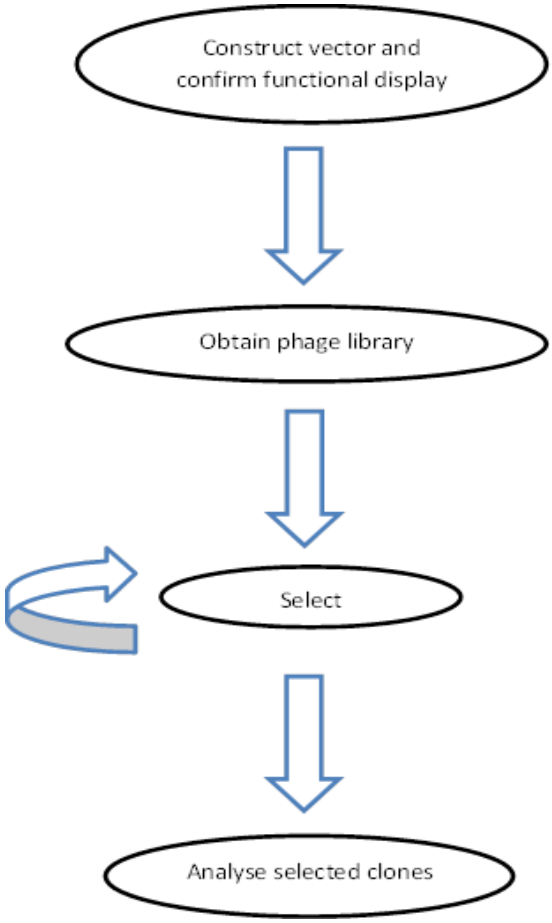

Figure 14. Flow diagram of doing a phage display project. At first, the vector is constructed, and the display system is confirmed. Then, the real Naïve library is built. After several rounds selection, selected clones are analysed. (Adapt from Russel et al., 2004)

In this work, the first stage is to construct a phagemid vector pAffi-100-Tryp and confirm the functional display of Z_{WT} and $Z_{Her2:342}$. The target combinatorial library has the cell size 4.7×10^{9} cfu which is achieved by randomization in 14 positions.

The variability of the library can be achieved by several methods. An equimolar mixture of the four activated nucleotides is used in the coupling step to produce a ran-

domized oligonucleotide sequence. However, 3 stop codons (TAA, TAG, and TGA) are possible to locate in the randomized position and induce prevention of the biosynthesis of the coat protein. An improved method is used where a mixture of guanine and thymine or guanine and cytosine are incorporated in position three of the codons. Two stop codons TAA and TGA are eliminated in this method. The remaining TAG can also be suppressed in a *supE E. coli* strain (Bossi, 1983). NN(G/T) degenerate codons are used for Affibody molecule library Zlib-1 and Zib2002 (Nord *et al.*, 1995; Grönwall *et al.*, 2007). They include 32 codons in total for all 20 amino acids. However, the best method to introduce variety in a library is the trinucleotide approach which was used in this research (Kayushin *et al.*, 1996). This approach eliminates the integration of stop and rare codons, which avoids some translational problems such as frame shifting (Belcourt and Farabaugh, 1990).

In some cases, it was possible to order appropriate pre-made phage display library, which are commercially available, to construct the phage library, such as Ph.D.™-12 Phage Display Peptide Library from New England Biolabs. The dodecapeptide (Ph.D-12) library has the complexities on the order of 10^9 independent clones. However, there is still no commercial Affibody molecule phage library. One quite useful Affibody molecule is Zlib2002 which was designed by Grönwall (Grönwall *et al.*, 2007). The new designed was based on the trinucleotide approach, and a protease sensitive site was introduced. The new Affibody molecule library with these improvements has the potential to become a promising tool for highly stringent binder selections.

The most common approach to select desired clones is through an *in vitro* binding incubation (Russel *et al.*, 2004). The members from phage library bind to a target. After washing, the retained phages are eluted. The process is also called biopanning. The eluted phages are used to re-infect *E. coli* for amplification, and several more stringent selections are performed. Generally, same sequencing fragment can be observed after three rounds selection. The first round of selection is considered the most important one, because any bias or loss of diversity will be amplified in the following rounds. The targets can be immobilized on streptavidin beads which was the approach used in this research. Furthermore, the targets can be adsorbed to plastic, and captured to agarose or plates (Christ, 2009). The enrichment of target specific phage is achieved by binding to immobilized target. After the selection, the eluted phage titrated and re-infected *E. coli*. New produced phage is isolated and purified for subsequent rounds of selection. After the third round selection, the individual colonies can be picked for binding affinity test. The fourth round selection with harsh conditions is performed to obtain binders with higher affinity. The enrichment of individual clones is also an indicator to monitor the selection process. Relative high enrichment means most of the phage particles before selection are able to bind to the immobilized targets. Therefore, relative low enrichment in the first two rounds and high enrichment in the third and fourth rounds may indicate a successful selection.

After three or four rounds of selection, the eluted phages and random clones are picked for post-selection analysis. The most common technique to determine the identi-

ty of the picked clones is sequencing. The binders with high repeat potentially have great binding properties to the targets. The candidate binders are selected for measuring the Affibody molecules properties. Biacore is one of the most popular biosensor on the interactions between proteins and other molecules. This technology is based on the label-free SPR which enables real-time detection monitoring of biological molecular events and provides quantitative information (Laure *et al*., 2001). ELISA is also a widely used approach to monitor the affinity of Affibody against the target (Bobrovnik, 2003). The binding properties are confirmed for future work.

5.2 Phagemid with full-length g3p and helper phage KM13

In this work, new phagemid was designed with full-length g3p. In previous researches, the low display efficiency is often observed with the conventional M13KO7 helper phage. The majority of progeny phage particles contain wild-type pIII which from helper phage, which causes high background of non-displaying phage which cannot participate in affinity selection (Bratkovič, 2010). Helper phage KM13 is used to reduce the background during the selection and the inactivated by trypsin. Helper phage KM13 is protease sensitive. This modified helper phage contains a modified g3p which encodes a trypsin cleavage site between D2 and D3. Therefore, after treated with trypsin solution, helper phage KM13 loses the ability to infect *E. coli* (Kristensen and Winter, 1998; Gotez *et al.*, 2002).

Full-length g3p is essential in phagemid vector, when helper phage KM13 is used. If the phage particles are produced from phagemid vector containing full-length g3p and helper phage KM13, the coat protein pIII is either full-length pIII with displayed desired Affibody molecule or full-length pIII without Affibody molecule. The pIII with Affibody molecule is originated from the constructed phagemid vector, and the Affibody molecule can be cleaved off from pIII with trypsin. On the other hand, the pIII without Affibody molecule is due to the helper phage KM13, and there exists a trypsin site between the D2 and D3 of pIII. With the treatment of trypsin solution, the pIII loses its infectivity on *E. coli*. Therefore, after treated with trypsin solution, the produced phage particle is only infective when it possesses pIII with displayed Affibody molecule. When truncated g3p is used instead of full-length g3p, the produced phage particles contain truncated pIII with Affibody molecule (from constructed phagemid vector) and full-length pIII without Affibody molecule (from helper phage KM13). However, with the treatment of trypsin, all the pIII only have D3 which means the phage particle is not infective. In the system of phagemid vector and KM13, high excess wild-type pIII is also expressed. However, it can be cleaved to lose all their domains D1 and D2 of pIII, thus the phage particles lose the ability to infect bacteria. The phage particle carrying the pIII from phagemid pAffi-100-Zlib-Tryp would retain their infectivity.

The helper phage KM13 has been well studies for antibody fragment produce. It is reported that better result was obtained when using KM13 helper phage in combina-

tion with trypsin treatment compared to other conventional methods. A large variety of antiidiotypic antibodies (Ab2s) are first time generated after only two or three rounds of selection (Goletz *et al.*, 2002). Furthermore, helper phage KM13 was used in constructing a compact phage display human scFv library. Good results were observed when using KM13 compared with regular helper phage M13KO7 (Pansri *et al*., 2009).

5.3 Library quality

Library quality is a key factor to get clones with desired properties. A library with insufficient size, poor or inappropriate diversity, or failed design is unlikely to get success. A highly diverse library is a good start for the selection. Also, it is necessary to check if the cloned genes are efficiently displayed on the phage capsid. Generally, the higher diversity of a library enables the higher possibility to attain high affinity binders.

The quality of an initial DNA-library is normally evaluated by it diversity. Because there are 20 different natural amino acids, in theory the number of different peptide sequences obtained by randomization of N residues can reach 20^N. If the number of random positions is 13, it is possible to get almost 1×10^{17} individual sequences. If one more random position is added, the number of obtained individual sequences can get 20 times amplified which is about 20×10^{18}. Although there were 14 positions randomized in this research, 18 different amino acids without Cys and Pro were distributed equally in the first 13 random positions. Moreover, in the last random position only Ile, Asp, His, Lys, and Tyr were used. Therefore, the maximum of obtained variants is about 8.3×10^{16}.

Generally, there are two factors limits the generation of different phage clones (library size). One is the amount of phage-encoding DNA molecules generated *in vitro*. The other one is the transformation efficiency of the DNA molecules into *E. coli*. It is considered that only 1% of the DNA molecule transferred into *E. coli* by electrotransformation. Several hundred μg of DNA are often required to obtain 10^9 transformants. Therefore, in this experiment, the efficiency of transforming the pAffi-100-Zlib-Tryp into the *ER2738* cell may limit the Affibody diversity. In order to increase the efficiency in each transformation, it is possible to choose the competent cell with good quality. Also, the size of the library size can be improved by increasing transformation times. Not all the clones display with similar efficiency. Some sequences are hard to display which causes underrepresentation in the phage library level. For an instance, cysteine is avoided in the new library, and the reason that the formation of inappropriate disulfide bonds can cause rare express as expected (Russel *et al.*, 2004).

A frequently used Affibody molecule phage library Zlib2002 contains 3×10^9. It was described by Grönwall (Grönwall *et al.*, 2007). 13 positions in the Z domain (Z_{WT}) were chosen for randomization through the NNG/T degeneracy. They were Q9, Q10, N11, F13, Y14, L17, H18, E24, E25, R27, N28, Q32 and K35. The Z domain-encoding DNA sequence with mutagenesis was inserted into phagemid vector pUC119, which resulted in the library vector pAffi1. Z domain-encoding DNA was linked to the gene

for 46 amino acid ABD and gene for the residues 249-406 of M13 phage coat protein III (g3p). In the end, the library Zlib2002 was generated by repeated electro-transformation with *E. coli*. The new library was designed based on the inspiration from Zlib2002. In my research, the mutations were introduced by trinucleotide approach. In the new constructed phagemid vector pAffi-100-Tryp, a full-length g3p took the place of the truncated one in phagemid pAffi1. Furthermore, phagemid pAffi-100-Tryp contained a trypsin sensitive site. The new library was also obtained through multi-transformation by electroporation. The size of the library was demonstrated to have 4.7×10^9 members after titration.

The distribution of each amino acid in every random position is an important factor to affect the library quality. After transformation, the phagemids from individual bacterial colonies are extracted for DNA sequencing. The frequency of appearance of each amino acid in the random position is calculated by analysis of the corresponding codon. In order to analysis the amino acid distribution of one library, at least 50-100 clones should be sequenced. In this research, 96 of clones originated from the transformed *E. coli* cell library stock and 96 of clones infected by the phage from prepared Affibody molecule phage library were randomly picked to determine the nucleotide diversity and frequency of the amino acid. However, sometimes it was impossible to analyze all the randomization position in the sequencing result from the first 96 samples. The reason might be that several phagemids entered the same cell during transformation. Therefore, prepared phage library enabled a better sequencing result.

5.4 Selection and post-selection analysis

Several parameters can influence the fate of affinity selection. The selection conditions were designed to obtain binders with high stringency. In the first round, the selection condition was performed to get the binders with lower stringency. Then, it is gradually intensified for higher stringency in the following selection rounds. For example, the target rhEphrin B3 concentration was decreased from 100nM to 1nM, where less targets available for phage binding leaded to higher stringency. Also, the washing times during the selection were increased from 3 to 10.

Furthermore, the number of phage copies of the initial phage library should be considered. Non-linear propagation of the individual clones clearly causes a significant bias in the selection procedures. Less than 1% of Affibody molecule from the initial library is expected to bind to target with specific binding in an idealized panning experiment. However, due to the unspecific interactions, phages can also be attached to the target with non-binding or unspecific binding. Though the non-binding phage can be eliminated under harsh washing condition, the unspecific binder might still exist. After the elution step, if the specific binders are present in a low number of copies and propagate slowly, they may have weak competence to outgrow compared to the unspecific binder. Therefore, a pre-selection was performed to overcome this problem. The unspecifically binding phage with a fast propagation can be significantly removed.

Elution condition is another important parameter affecting the outcome of affinity selection. The wildly accepted non-specific elution is using buffer 0.2 M Glycine-HCl (pH 2.2). The release achieved by weakening the peptide-target interaction instead of destroying phage infectivity. However, it is unable to break some interactions where binders having great affinity. The trypsin digestion used in this work has been reported as a favorable method for releasing affinity-selected virions from immobilized selectors (Thomas and Smith, 2010).

As shown in the sequencing result of Table 5, some similar patterns were found among the binders. Interestingly, only Ile was found in Pos.31 in all the sequenced phagemid. Pos.31 is considered as a core position in the interaction between the Affibody molecule and target. In the Pos.31 randomization design, several kinds of amino acids were chosen to vary the properties of engineered Affibody molecules. Ile is present in the Z_{WT} residue; Asp is with negative charge; Lys is with positive; His switches at pH5.5; Tyr is often included in antibody-antigen interaction surfaces.

However, quite high amount of phages display Z_{WT} was observed (Figure 5). The contamination was suggested due to the pre-selection system where ENBREL® was used. Because ENBREL® is a Z_{WT} specific protein, Affibody molecule Z_{WT} was bound to biotinylated ENBREL® in the pre-selection. Though the complex Affibody molecule and ENBREL® was immobilized on the beads with streptavidin, it was still possible to transfer beads with Z_{WT}-displaying phage into a new Eppendorf tube for the selection against rhEphrin-B3. The phage particles displaying Z_{WT} was eluted off with other phage expressing specific Affibody molecule for rhEphrin-B3. Since the pre-selection was performed in each round before the selection against rhEphrin-B3, the enrichment of Z_{WT} displaying phage could result in a high repeat shown in the sequencing result. Altering the target protein in different selection rounds or using other immobilization method instead of the interaction between EZ-Link TM Sulfo-NHS-LC-biotin and Dynabeads ® M-280 Streptavidin will be helpful to decrease the bias.

Biacore results showed several most promising Affibody molecule was from Track 4 (Figure 8). However, it only showed the specificity analysis due to the time restriction. In order to determine the affinity, dissociation constant K_d should be measured in further researches. Kinetic analysis is also necessary to study the binding properties of selected Affibody molecules (Karlsson and Fält, 1997; Leonard *et al.*, 2011).

6. SUMMARY AND FUTURE WORK

In this project, a new Affibody library has been constructed for highly stringent binder selections. The size of the library is approximately 4.7×10^9 cfu, and there were 76% functional clones in the library. In the selection to rhEphrin-B3, the bound phage was able to be cleaved by trypsin, and reinfect. After four rounds selection, candidate binders were selected. According to the sequencing results, there were similar patterns among the candidate binders. Also, only Ile was found in Position 31.

In the future work, more protein needs to be expressed. With the sufficient amounts of proteins, the affinity of promising binder will be measured. Since rhEphrin-B3 is involved in the interactions with Eph receptors, the Affibody binding to rhEphrin-B3 but not Eph receptors can be selected. Furthermore, this is a cooperative project with Karolinska Institute. This library may be used for selecting other targets of interest.

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APPENDIX

Sequence of Tri-121-mer

5’-ACAACAAATTCAACAAAGAAAXXXXXXGCGXXXXXXGAGATCXXXXXX
CTGCCGAACCTGAACXXXXXCAAXXXXXXGCCTTCXXXXXXAG-
TTTAXXXGATGACCCAAGCGAAAGCGC-3’

Trinuclotiders (Trimers) used in Tri-121-mer

Trimer	Amino Acid
AAA	Lys
AAC	Asn
ACT	Thr
ATC	Ile
ATG	Met
CAG	Gln
CAT	His
CCG	Pro
CGT	Arg
CTG	Leu
GAA	Glu
GAC	Asp
GCT	Ala
GGT	Gly
GTT	Val
TAC	Tyr
TCT	Ser
TGC	Cys
TGG	Try
TTC	Phe

Peptide sequence of protein 3

AETVE SCLAK PHTEN SFTNV WKDDK TLDRY ANYEG CLWNA TGVVV CTGDE TQCYG TWVPI GLAIP ENEGG GSEGG GSEGG GSEGG GTKPP EYGDT PIPGY TYINP LDGTY PPGTE QNPAN PNPSL EESQP LNTFM FQNNR FRNRQ GALTV YTGTV TQGTD PVKTY YQYTP VSSKA MYDAY WNGKF RDCAF HSGFN EDPFV CEYQG QSSDL PQPPV NAGGG SGGGS GGGSE GGGSE GGGSE GGGSE GGGSG GGSGS GDFDY EKMAN ANKGA MTENA DENAL QSDAK GKLDS VATDY GAAID GFIGD VSGLA NGNGA TGDFA GSNSQ MAQVG DGDNS PLMNN FRQYL PSLPQ SVECR PFVFS AGKPY EFSID CDKIN LFRGV FAFLL YVATF MYVFS TFANI LRNKE S